

A STUDY ON USTILAGO VIOLACEA (PERS.) FUCKEL  
ON SILENE DIOICA (L.) CLAIRV

Abdel Gadir Hassan

A Thesis Submitted for the Degree of PhD  
at the  
University of St Andrews



1965

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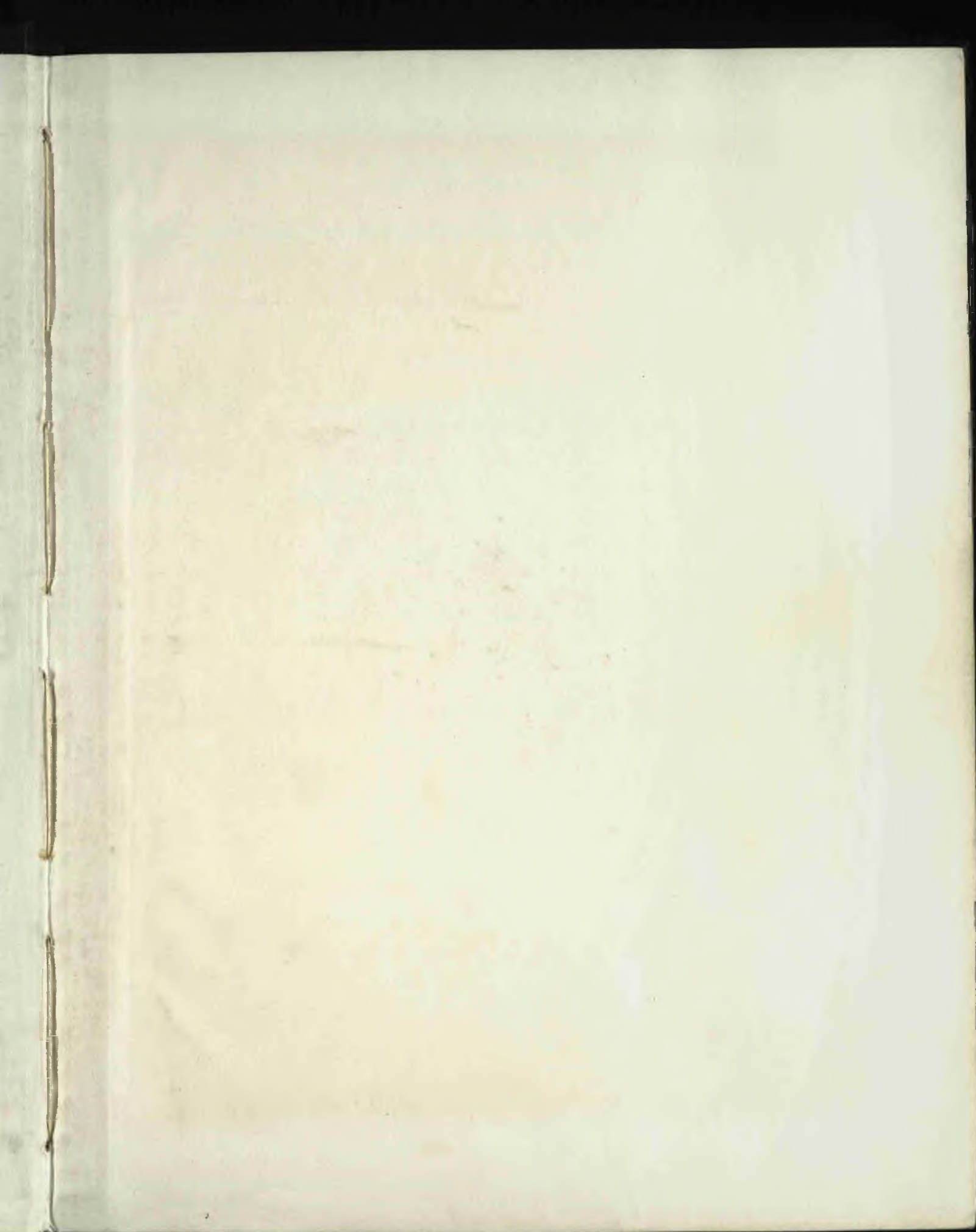
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DECLARATION.

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I hereby declare that the following Thesis is based on  
a record of work done by me, that the Thesis is my own work  
and that it has not previously been presented for a  
Higher Degree.

SILENE DIOICA (L.) CLAIRV.

The research was carried out in the Department of Botany at  
St. Salvador's College of the University of St. Andrews under  
the direction of Professor J. A. Macdonald.

by

Abdel Gadir Hassan, B.Sc.

A thesis submitted to the University of St. Andrews for the  
degree of Doctor of Philosophy.

Department of Botany,  
University of St. Andrews.

November, 1965.



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CAREER.

DECLARATION.

I graduated from the University of St. Andrews in June, 1954 with a second class honours in Botany. From July, 1954, to September, 1955, I served as a Research Assistant in the Department of Botany at the University of St. Andrews.

I hereby declare that the following Thesis is based on a record of work done by me, that the Thesis is my own composition, and that it has not previously been presented for a Higher Degree.

In September, 1955, I was awarded a British Government Scholarship, to do research <sup>out</sup> in the Department of Botany at the University of St. Andrews. The research was carried out in the Department of Botany at St. Salvator's College of the University of St. Andrews under the direction of Professor J. A. Macdonald.

I was admitted as a Research Student.

During the tenure of the Scholarship the research work presented here is the result of my own work.

AS  
A.G. JASSAN



### CAREER.

#### CERTIFICATE.

I graduated from the University of St. Andrews in June, 1954 with a second class honours in Botany. From July, 1954, to September, 1962, I served as a Secondary School Science Master in the Sudan.

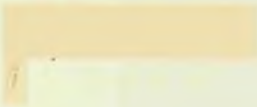
In September, 1962, I was awarded a Sudan Government Scholarship, to do research in Botany at the University of St. Andrews.

I matriculated at the University of St. Andrews and was admitted as a Research Student under Ordinances 16 and 61.

During the tenure of the Scholarship, I undertook the research work presented here for the Ph.D. degree.

CERTIFICATE.

I certify that Abdel Gadir Hassan has spent nine terms of research work under my direction and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews), and that he is qualified to submit the accompanying Thesis in application for the degree of Doctor of Philosophy.





## CONTENTS

### INTRODUCTION.

### ACKNOWLEDGEMENTS

I wish to record my indebtedness to Professor J. A. Macdonald of the Department of Botany, St. Salvator's College, for supervising the work presented in this Thesis, and for the continued interest he has shown in the investigations.

|                                                   |    |
|---------------------------------------------------|----|
| Spore germination .....                           | 11 |
| Reproduction of <i>Silene elaeagnifolia</i> ..... | 12 |
| Sexuality in <i>Silene</i> .....                  | 13 |

### MATERIALS AND METHODS.

|                                                         |    |
|---------------------------------------------------------|----|
| Sources of plants and spores .....                      | 14 |
| Field observations on <i>Silene elaeagnifolia</i> ..... | 15 |
| Sex ratios .....                                        | 16 |
| Root tip squashes .....                                 | 17 |
| Inoculation experiments .....                           | 18 |
| Seed inoculation .....                                  | 19 |
| Flower infection .....                                  | 20 |
| Seed infection .....                                    | 21 |
| Seedling infection .....                                | 22 |

CONTENTS

INTRODUCTION.

|                                                |    |
|------------------------------------------------|----|
| General .....                                  | 1  |
| Nomenclature of the Fungus .....               | 4  |
| Host Plants .....                              | 5  |
| Life History of <i>Ustilago violacea</i> ..... | 7  |
| Spore Germination .....                        | 9  |
| Spore Formation .....                          | 11 |
| Nomenclature of <i>Silene dioica</i> .....     | 12 |
| Sexuality in <i>Silene</i> .....               | 14 |

MATERIALS AND METHODS.

|                                             |    |
|---------------------------------------------|----|
| Sources of Plants and Spores .....          | 26 |
| Field observations on diseased plants ..... | 27 |
| Sex ratios .....                            | 28 |
| Root tip squashes .....                     | 30 |
| Inoculation experiments .....               | 34 |
| Seed transmission .....                     | 34 |
| Flower infection .....                      | 36 |
| Bud infection .....                         | 37 |
| Seedling infection .....                    | 40 |



## INTRODUCTION

### GENERAL

#### Spore germination

Germination in water and nutrient media .....43

Effect of temperature on germination .....47

Age and storage of spores .....50

Effect of pH. on germination .....53

Sporidial fusions .....55

Cytological methods on Fungus .....59

Parasitic mycelium .....68

Observations on infected flowers .....74

Stability of sex in *Silene* .....78

Emasculation experiments .....81

Microscopy, Photography and Microphotography ....83

Discussions and Conclusions .....84

Summary .....94

Bibliography .....98

Caryophyllaceae are attacked by the fungus Ustilago violacea (Pers.) Fuckel., the flowers become pseudohermaphrodites. The question arises as to whether it is the female flower that becomes hermaphrodite, or the male one or both.

## INTRODUCTION

### GENERAL

Ustilago violacea (Pers.) Fuckel., has for a considerable time been known as a pathogen of quite a number of plants belonging to the family Caryophyllaceae. The first reference to this fungus according to Liro (1924), is that Linnaeus (1737), observed the smut induced <sup>anthers</sup> on normally female flowers of the "white campion", and thought perhaps he had a hermaphrodite form of a normally dioecious plant. Later on, in 1755, Linnaeus considered this hermaphrodite plant as a form of Lychnis dioica L., but still did not mention ~~as~~ having noticed the fungus in the induced anthers.

Exactly who first discovered this phenomenon as smut induced, is not known as a certainty. However, in his Synopsis Methodia Fungorum, page 225, Persoon (1801) describes Uredo (Ustilago) violacea, for which he gave Saponaria officinalis and Silene nutans as host plants. But he gives no mention of the relation between the fungus and induced hermaphroditism. Giard (1888) gives the credit of the discovery of this relationship to Tulasne (1847). But of course, the gap between Linnaeus and Tulasne is rather big. Liro (1924) reports that in later years the names Ustilago violacea Pers., and Uredo antherarum Fries, were usually given to the species that produces its brand spores on the anthers of a number of plants in the family Caryophyllaceae.

Earlier workers have known this fungus under quite a number of different names, which were sometimes based on the host plants.

Various workers since the time of Linnaeus have reported the fact that when Silene dioica (L.) Clairv. and other related members of the family

even before the male are opened and the spores are exposed. This is because of the general bushy appearance of the whole plant, and the thick



Caryophyllaceae are attacked by the fungus Ustilago violacea (Pers.) Fuckel., the flowers become pseudohermaphrodites. The question arises as to whether it is the female flower that becomes hermaphrodite, or the male one or both.

Giard (1888) gave such an effect of the fungus on its host plant the term "Parasitic castration". He recognised three types of castration:

Androgene, when it induces into the female, characters which are normally considered as belonging to the male..

Thelygene, when the opposite of the above occurs.

Amhigene, when the characters are mixed.

Giard considered that Ustilago violacea represents an example of the first type. This means that he was led to believe that it is the female Silene which undergoes such a change as a result to the fungal attack.

Vuillemin (1891), [in Fischer (1960)], states that, "besides the parasitic castration there is a hypertrophy of the anthers ..... This hypertrophy gives the infected flowers a monoclinal appearance."

MacMillan (1892) reports that as a result of the infection, of female plants, the ovary aborts, since the nutriment that should normally go to the ovary is now shared by the hypertrophied anthers. Furthermore, he adds, that other accessory characters of the staminate flower are developed in the proper order, so that the normally pistillate but apparently staminate flower, closely resembles a pollen bearing flower to the extent that it is no doubt visited by the same pollinating insects.

Ustilago violacea, apart from this parasitic castration, has some other noticable effects on its host plant, and especially on the flower.

Giard (1888) noted that not only do well developed stamens appear in the female flower, but that when the male flower is infected, an ovary though not quite well formed is observed. The ovules in the abortive ovary in the infected female never develop into seeds.

White (1936) reports that an infected plant is easily recognisable even before the buds are opened and the spores are exposed. This is because of the general bushy appearance of the whole plant, and the thick

and squat buds.

Baker (1947) noted that an infected flower can be detected before its bud opens, because of the irregular shape and split calyx. He attributes the split calyx to the great elongation of the claws of the petals inside the bud. Also the filaments elongate appreciably causing a bulge to one side of the bud.

Spencer and White (1950) noticed that infected plants are weakened and stunted, and that the axillary growth is so profuse that it gave the plant a rather bushy appearance. The number of flowers per branch is also much greater, but the flowers are generally smaller and paler in colour.

The effect of the fungus on its host plants, that is, induced hermaphroditism, has been a subject of great interest to those working on the genetics and sexuality of dioecious plants. This will be discussed in the coming pages.

6. Saxosa antheraria Schlechtendal 1821

7. Detilago antheraria Pries 1833

8. Krysiha antheraria Wallroth 1855

9. Microbotryum antheraria Leveillé 1847

10. Detilago violacea Puckel 1861

Lira (1934) in his Detilago violacea split Detilago violacea into seven species. These are:



NOMENCLATURE:

## SYNONYM:

## AUTHORITY AND DATE

## QUOTED BY

- |                                   |                    |                                                                                  |
|-----------------------------------|--------------------|----------------------------------------------------------------------------------|
| 1. <u>Uredo violacea</u>          | Persoon 1801       | Plowright 1889<br>Fischer 1951                                                   |
| 2. <u>Farinaria Stellarie</u>     | Sowerby 1803       | Saccardo 1888<br>Plowright 1889<br>Ainsworth and<br>Sampson 1950<br>Fischer 1951 |
| 3. <u>Uredo antherarum</u>        | De Candole 1815    | Saccardo 1888<br>Plowright 1889<br>Ainsworth and<br>Sampson 1950<br>Fischer 1951 |
| 4. <u>Caeoma violaceum</u>        | Nees 1817          | Saccardo 1888<br>Fischer 1951                                                    |
| 5. <u>Caeoma violacea</u>         | Martius 1817       | Saccardo 1888<br>Fischer 1960                                                    |
| 6. <u>Caeoma antherarum</u>       | Schlechtendal 1824 | Saccardo 1888<br>Fischer 1951                                                    |
| 7. <u>Ustilago antherarum</u>     | Fries 1832         | Saccardo 1888<br>Plowright 1889<br>Ainsworth and<br>Sampson 1950<br>Fischer 1951 |
| 8. <u>Erysibe antherarum</u>      | Wallroth 1833      | Saccardo 1888<br>Fischer 1951                                                    |
| 9. <u>Microbotryum antherarum</u> | Leveille 1847      | Saccardo 1888<br>Fischer 1951                                                    |
| 10. <u>Ustilago violacea</u>      | Fuckel 1861        | Saccardo 1888<br>Plowright 1889<br>Ainsworth and<br>Sampson 1950<br>Fischer 1951 |

Liro (1924) in his Ustilagineen Finnlands split Ustilago violacea into seven species. These are:

1. Ustilago Lychnis-dioicae.

2. Ustilago dianthorum.

3. Ustilago superba.

4. Ustilago coronariae.

5. Ustilago stellaria.

6. Ustilago silene-nutantis.

7. Ustilago silene-inflatae.

#### HOST PLANTS.

Saccardo (1888) gives a long list of plants all belonging to the family Caryophyllaceae as being host plants to the fungus Ustilago violacea (Pers.) Fuckel. Plowright (1889-92) adds a few more species to Saccardo's list. Liro (1924), White (1936), and Ainsworth and Sampson (1950) all added more species of host plants. The list as it stands now includes more than thirty species mostly from the Caryophyllaceae:

- |                                    |                                  |
|------------------------------------|----------------------------------|
| 1. <u>Silene quadrifa</u>          | 2. <u>Silene otites</u>          |
| 3. <u>Silene nutans</u>            | 4. <u>Silene inflata</u>         |
| 5. <u>Silene rupestris</u>         | 6. <u>Silene chloranthae</u>     |
| 7. <u>Silene maritima</u>          | 8. <u>Silene pratense</u>        |
| 9. <u>Silene alba</u>              | 10. <u>Silene vulgaris</u>       |
| 11. <u>Silene dioica</u>           | 12. <u>Silene noctiflora</u>     |
| 13. <u>Silene acaulis</u>          | 14. <u>Silene alsine</u>         |
| 15. <u>Silene cucubalus</u>        | 16. <u>Silene Antirrhina</u>     |
| 17. <u>Lychnis flos-cuculi</u>     | 18. <u>Lychnis vespertina</u>    |
| 19. <u>Lychnis coronariae</u>      | 20. <u>Dianthus deltoides</u>    |
| 21. <u>Dianthus carthusianorum</u> | 22. <u>Dianthus gracialis</u>    |
| 23. <u>Dianthus caryophyllus</u>   | 24. <u>Dianthus superba</u>      |
| 25. <u>Dianthus morspessulanus</u> | 26. <u>Saponaria officinalis</u> |
| 27. <u>Stellaria graminea</u>      | 28. <u>Stellaria holostea</u>    |
| 29. <u>Arenaria serpyllifolia</u>  | 30. <u>Cucubalus baccifer</u>    |
| 31. <u>Cerastium viscosum</u>      | 32. <u>Vicaria vulgaris</u>      |
| 33. <u>Agrostemma githago</u>      | 34. <u>Malachia aquatica</u>     |
| 35. <u>Pinguicula alpina</u>       |                                  |



The last species listed here does not belong to the family Caryophyllaceae, but is included by Saccardo and MacMillan as a host plant.

It was this long list of host plants that prompted Liro (1924) to investigate the validity of the assumption that the chlamydospores formed on the anthers of all these plants belonged to one and the same species of anther smut- namely Ustilago violacea. He reports that:

1. The chlamydospores taken from Dianthus carthusianorum, and Saponaria officinalis showed some differences in their germinations.
2. Spores from Dianthus deltoides were bigger and darker than spores from other species.
3. Schroeter (1877) had often observed groups of healthy Silene (species not specified), growing among large patches of infected Dianthus deltoides plants. Liro himself also gives a similar example of healthy Stellaria graminea growing among heavily infected Dianthus deltoides plants.

Counting on these racial and physiologic specializations, Liro (1924), decided that what is generally known as Ustilago violacea (Pers.) Fuckel., is in fact a composite species, and should, therefore, be broken up into as many species as there were host plants. But actually he recognised only seven such forms.

Despite the strong arguments put forward by Liro for the break up of Ustilago violacea, he quotes Zillig (1921) as having successfully infected Agrostemma githago and Silene noctiflora, with chlamydospores taken from Silene alba.

the infection really originated from the presumed infected seeds? Becker (1907), according to Baker (1945), tried over several years to repeat this experiment, but had no positive results. I have, in fact tried



LIFE HISTORY OF USTILAGO VIOLACEA (PERS.) FÜCKEL.

The life history of this fungus has been worked out by a number of mycologists, and with slight variations in their reports. The more important contributions in this line were by Plowright (1882 and 1889), Brefeld (1883), de Bary (1884), Dangeard (1892), Harper (1900), Baar (1903), Liro (1924), Goldschmidt (1928) and Wang (1932). Other workers made some contributions on certain aspects of the life history of this fungus.

It might be more appropriate to start discussing the life cycle with the resting spore, since it is the one stage that one encounters in the field. Plowright, Dangeard, Liro and Sampson and Ainsworth, all agree on their descriptions of the resting spore as being spherical or nearly so, with a pale violet tint. The powdery mass of spores has a pinkish-purple colour. The average diameter of the spore  $7-8\mu$ , while the reticulations on the wall measure about  $1\mu$ .

It is now established that when Silene dioica and other related species get infected with Ustilago violacea, the anthers, whether naturally existing, as in the male flower, or induced, as in the female, become filled with smut spores instead of pollen. The question arises as to how these spores get transferred from one plant to another, or even from one flower to another.

Brefeld (1905), [according to Liro (1924)], advocated seed transmission of the disease. This of course implies that such seeds carrying the mycelium or even just the spores must come from infected female plants. But my detailed examinations of infected female plants has shown beyond doubt, that ovaries in such plants never produce seeds. Brefeld, however, according to Liro (1924), dusted spores of Ustilago on the stigmas of Silene alba, collected the seeds from such treated plants, and raised plants from them. He claims to have obtained 20% infections. But has the infection really originated from the presumed infected seeds? Hecke (1907), according to Baker (1945), tried over several years to repeat this experiment, but had no positive results. I have, in fact tried



various ways of raising infected plants from smut treated seeds but without success. It appears that seed transmission of this disease should be ruled out.

MacMillan (1892) states that "....a normally pistillate, but apparently staminate flower presents the appearance of pollen bearing to such an extent that it is doubtless visited by those insects which habitually transfer pollen from the staminate to the pistillate flowers. This is of a clear advantage to the fungus, for it is thus sown upon young buds as well as upon stigmatic areas...."

Plowright (1892), having watched the growth of infected plants, was "....struck by the number of bees which visited the diseased blossom and it occurred to my mind that these insects might be the means of conveying it from one plant to another...."

Eriksson (1930) states that the spores are transported usually in the night, between 8 and 11 p.m., by insects having a long proboscis, to the stamens and styles of other flowers, and perhaps by wind to seedlings and young shoots.

Leach (1940) thinks that this disease is a striking illustration of the adaptation of the fungus to insect transmission.

Fischer (1957) reports that Maurizio (1941), in a study of the fungus spore content of mountain honey, captured ten bees that had visited infected Silene alba plants, and found that in the nine bees where the vesicles contained honey, there were spores of Ustilago violacea, and that spores were also found adhering to the hairs on the bees' legs.

Plowright (1892) having noticed insect visitors to the infected as well as the healthy flowers, was prompted to try blossom infections which he did with a certain amount of success.

Insect transport of the spores is perhaps one of the stages in the transmission of the disease, and could be paralleled with pollination as



being just one step towards fertilization. However, this is not the only method whereby spores are transported. Wind cannot be excluded as an agent.

Werth (1911) working on the possibility of flower infections, dusted smut spores on both male and female flowers. After eight weeks, infected blossoms appeared on the same branches where inoculations were carried out, and later on, such blossoms appeared in neighbouring branches.

Hecke (1926) [in Fischer (1957)] developed the theory of shoot infection. In the fall, he removed the soil from around two year old plants of Silene alba, thus exposing the crown which he covered with a mulch of Ustilago violacea spores. The next spring he had many infected plants. He therefore concluded that the young shoot must be the seat of infection. He then inoculated some plants in the axillary buds, and kept them under very humid conditions for some weeks. He claimed 100% infections.

Gaumann (1950) believes that Ustilago violacea presents a good example of seedling infection. But he adds that shoot infection also takes place, entrance being made through young tissues, buds, and growing shoot.

Horsfall and Diamond (1959) give Ustilago violacea as an excellent example of a fungus infecting its host plant through flowers, buds, and excised stems.

Gram and Weber (1951) report that the host plant receives infection through spores falling on the axillary buds.

#### SPORE GERMINATION

The resting spore or chlamydospore, germinates by a germ tube after a very short immersion in water. In nature this water is trapped in the axils of leaves where the buds are, or inside the flowers. The germ tube or promycelium as de Bary calls it, is ideally formed of three cells.

Plowright (1889), Brefeld (1883), de Bary (1884), Harper (1900), and Wang (1932) have all noticed that, sometimes two promycelia come from the same spore, either from the same spot or from two different points, but simultaneously. Normally, a second promycelium comes out after the first one has fallen off.



The promycelia in Ustilago violacea do not develop directly to produce hyphae, as in some other related smut fungi, but they bud off basidiospores from their three cells. These basidiospores are known as sporidia. When the promycelium is mature, it falls off, and is replaced by a second one, a third, and in fact a succession of these promycelia is repeated for some time.

Dangeard (1892) examined the resting spore, the sporidia, and the promycelial cells cytologically. He found that all are uninucleated. My own observations, to be discussed later on, show that the resting spore has a diploid nucleus, which divides meiotically. The promycelial cells and the sporidia being haploid.

The fact that more than one promycelium come out from the spore at the same time is a clear indication that the division takes place within the chlamydospore and at the onset of germination. Wang (1932) confirms this and adds that generally one nucleus goes into the promycelium before the second meiotic division. She found that the haploid number of chromosomes is two.

When the sporidia are placed in nutrient media, they keep on dividing indefinitely, forming yeast-like colonies. But when placed in water, or when the nutrient medium is exhausted, that process stops. Plowright gives 24 hours as the average time for budding to stop when the spores are germinated in water. Dangeard (1892) gives a similar description to the yeast-like budding of sporidia. Harper (1900) adds that these sporidia produce light rose coloured colonies on solid media.

When the sporidia are starved, or when the chlamydospores are simply germinated in water, and left there for more than two days, they start to unite in pairs. The fusion of sporidia in Ustilago violacea (and other species), has been the cause of the reported controversy between Brefeld and de Bary. De Bary referred to this type of fusion as a form of sexual union, and called it conjugation. The idea of such fusions being referred



to as sexual was strongly objected to by Brefeld according to de Bary. Brefeld argues that although such fusions did occur always between only two sporidia, it occurred also between sporidia and promycelial cells. And if those sporidia were to be looked upon as gametes, they should not have kept on dividing in nutrient media. Their fusions according to Brefeld should be considered as a sort of vegetative coalescence similar to that of hyphal anastomosis.

Goldschmidt (1928) recognised two mating types of sporidia which may unite in one of two ways. Either two sporidia of the different mating types come together under the proper environmental conditions, their cell walls at the points of contact dissolve and a copulation hypha is formed, or each cell would send off a copulation hypha towards the other, and the two hyphae coalesce. This form of bi-polar heterothallism, as it is now known, and reported for this species by Goldschmidt, is supported by Spencer and White (1950), who added that two sporidia having had their origin from the same promycelial cell would not unite with each other, nor would they fuse with their parent cell.

After fusion of these two different sporidia, according to Dangeard (1892), the infective hypha is formed, or the dikaryotic stage commences. Baar (1903) has followed the course of the mycelium in Silene pratense, where he found it to be systemic and overwintering in the rootstocks. He found great difficulty in tracing individual hyphae, since the mycelium was so interwoven, that it looked to him like some intensely coloured mass of threads running parallel to the vascular tissues. Wang (1932) reports that the mycelium in the host tissues is not uniformly dikaryotic. Binucleate cells were formed only at the last stages preceding spore formation.

#### SPORE FORMATION

After establishing itself in the host tissues, the dikaryotic mycelium keeps pace with the growing points of the young shoots, and invades the hypertrophied anthers. Dangeard (1892) states that the mycelium becomes



twisted round itself and forms finally sporogenous balls in the pollen sacs after having destroyed the pollen mother cells. This was supported by Plowright in the same year, and adds, that the spore forming hyphae enlarge greatly, and their interior gelatinizes to the extent their lumens are obliterated and the various hyphae become entangled and appear as though they were glued together. The hyphae become specially thicker at certain points, which later on develop into spores. De Bary (1884) previously noticed that when the hyphae are gelatinized, they are segmented into short isodiametric members. The protoplasm in each such member is surrounded with a thick hyaline gelatinous sheath inside which the spore wall is laid down.

Dangeard (1892), examining infected anthers, reports that a section through very young stamens would show a mass of undifferentiated parenchyma with traces of intercellular mycelium at the corners of the anther chambers. Later on this mycelium completely occupies the centre of the anther lobes. From then onwards, the mycelium develops more round the cells filling the intercellular spaces and eventually destroying the cells.

The small segments to which the hyphae broke up were seen by Dangeard, each to contain two nuclei. Those two nuclei fused while the thick episporous wall was being laid down. Thus according to Dangeard, the young spore has two nuclei, while the mature spore has but one nucleus. Dangeard refers to the mature spore as an oogonium, enclosing an oospore resulting from the fusion of a male and a female nucleus. The only real diploid stage in the whole life history of Ustilago violacea is the resting spore or chlamydospore.

Looking at the list of host plants of the fungus Ustilago violacea, one finds Silene dioica being included as one of the species in the genus Silene which is attacked by the fungus. The fact that it is a dioecious member of the genus, made the study of the fungal attack on it more interesting, mainly because of its reported effect on sexuality.

Silene dioica (L.) Clairv. and other related species, belong to the



genus Silene of the Family Caryophyllaceae. The genus has in fact, been the subject of a lot of controversy among the various workers. This is reflected in the uncertain position of the "Red Champion", which has been referred to as belonging to the genera Silene, Lychnis, and Melandrium.

The classification proposed by Chawdhuri (1957) is the one adopted in the most recent floras.

The species - Silene dioica(L.) Clairv., is referred to in the literature under quite a number of synonyms. The more important of these are:-

Lychnis dioica.L.

Lychnis diurna. Sibth.

Melandrium dioicum(L.) Coss and Germ.

Melandrium rubrum (Weigel) Garke.

Melandrium silvestre (Schkuhr.) Röling.

In his analysis of the subtribe Silenoideae, Chawdhuri (1957) retained Lychnis as a distinct genus from Silene, since in Silene the capsule valve is split (as is the case in the red champion), while it is always entire in Lychnis. With regard to Silene and Melandrium, Chawdhuri argues that many of the distinctive characters shown by Melandrium, such as the inflation of the calyx and the duplication of the nerves, are shared by many members of the genus Silene. Moreover, Chawdhuri, refers to the natural hybridizations between Silene viscosa and Melandrium dioicum, and between Silene viscosa and Melandrium pratense, and some other hybridizations between species in these two genera, and considers that all species previously put in the genus Melandrium can very comfortably be accommodated in Silene, and so Silene and Melandrium should be treated as one and not two separate genera. Thus the Red Champion gets its correct name - Silene dioica(L.) Clairv.

This name is adopted by Dandy (1958) in his "List of the British Vascular Plants", Perring and <sup>Walter</sup> Smith (1962) in their "Atlas of the British Flora", <sup>Clapham</sup> Tutton and co-authors (1962) in "Flora of the British Isles", and in <sup>Tutin et al</sup> their more recent work, (1964) "Flora Europea".

Blackburn (1903) investigated the system of many dioecious light plants with the intention of throwing some light on the matter of sex chromosomes in plants. One of her experimental plants was Silene dioica.



The Red and the White Campions have attracted much attention since the latter half of the last century and all through this century, mainly because of the phenomenon of separate male and female plants exhibited by these two species. Lewis (1942) reports that in all the Angiosperms, only about 5% of the families are completely dioecious, and in his analysis of the British flora, he states that out of 91 families only 6 are completely dioecious, and at the generic level, the figures are 15 out of a total of 523 genera.

Work on both the Red and White Campions, which will be discussed later on, revealed that there are easily recognisable male and female chromosome complements. This triggered off further investigations on how sex is determined in dioecious plants.

Shull (1910, 1911 and 1912) made a series of genetical investigations on Silene dioica - referring to it as Lychnis dioica. He reports that hermaphrodite mutants of Silene dioica occur in pure bred individuals at a rate of 0.1% and that none of these hermaphrodites are diseased. Some of these are actually functional males or females. The hermaphrodites of Shull were believed by him to be modified males, for in all the families where these were the pollen donors, the offspring showed the same ratios of males to females as would have been expected had a normal male been used as pollen parent. He draws attention to psuedo-hermaphrodites reported by Strasburger, resulting from the attacks on Silene dioica by the smut Ustilago violacea, pointing out that these hermaphrodites are non-functional, since the pollen is replaced by the smut spores. He considers that this justifies the theory that each sex has the potentialities of the other, and that sexes in fact represent alternative states which in different species may be attained in different ways, through addition, subtraction, or transformation, and that environment plays a very important part here. Shull comes to the conclusion that sex reversal in Silene due to the smut is only somatic.

Blackburn (1923) investigated the cytology of many dioecious higher plants with the intention of throwing some light on the matter of sex chromosomes in plants. One of her experimental plants was Silene dioica



(under the name Melandrium dioicum). She found that in both the male and the female plants, there were 24 somatic chromosomes; ~~of~~ these two were clearly larger than the rest. In meiosis, she noticed that in the female, the two large chromosomes were of the same size, whereas in the male, one of these chromosomes was appreciably larger and different in shape from the other. The larger one looked like a hockey stick, while the shorter one was pear shaped and about  $\frac{2}{3}$  the length of the other.

These findings of Blackburn were confirmed in Japan by Ono (1939). He referred to his plant as Melandrium dioicum. Ono used the Colchicine method of inducing polyploidy. It was ascertained by him that the diploid number of chromosomes in Silene dioica is 24, and that the female complement is  $22+X+X$ , and that the male complement is  $22+X+Y$ . In other words it is the male which is heterozygous. These X and Y are the sex chromosomes.

Work by Ness (1961) on both the Red and the White Campions, confirmed that the diploid number in Silene is 24, and that the female complement is  $2A.+XX$ , and that the male complement is  $2A.+X+Y$ .

Warmke and Blakslee (1939), confirmed that the Y-chromosome is the larger one of the two sex chromosomes. They arrived at this conclusion after crossing polyploids with the complements  $4A.+XXXX$  and  $4A.+XXYY$ . Various combinations of Xs and Ys were obtained. In a plant with three sex chromosomes of one size and a fourth of a different size; the three similar ones from such a cross must be X-chromosomes, since it is impossible to obtain a  $4A.XYYY$  from a cross between  $4A.+XXXX$  and  $4A.XXYY$ . It was thus possible for these two workers to estimate the relative size of the two sex chromosomes within a cell. They reported that in all the material they dealt with, the Y-chromosome was the larger one.

Warmke (1946) discussed the sex potentialities in both dioecious plants and animals. He states that in most cases the female is homozygous and the male is heterozygous. This is also the case in Silene; where the female produces only one type of gamete with X-chromosomes only, while the male produces gametes with either X or Y-chromosomes.



Warmke's experiments were carried out to give evidence for the location of the male and female determining genes, and their relative strength in Silene. He raised polyploids using the colchicine method, where seeds were soaked in a 0.05 - 0.4% aqueous solution for one to four days, the seeds were then germinated and root tips were taken out and used in studying the roles of the X, the Y, and the autosomes. He used the Feulgen stain for the root tips and acetocarmine for studying meiosis in the anthers.

In determining the role of the Y-chromosome, Warmke used two tetraploid plants having the complements:

- (a) 4A.XXYY which was a male, and
- (b) 4A.XXXX which was a female, and crossed the two.

He got 3.5% plants with the complement 4A.XXXX which were all females and similar to the maternal parent, and 3.5% plants with the complement 4A.XXYY which were all males and similar to the paternal parent. 89% were plants with the complement 4A.XXXY which were all males, but with the occasional hermaphrodite blossom. The remaining 4% being plants with three or five sex chromosomes. Among the plants with three sex chromosomes, there was one with 4A.XXX which was a strong female. When this plant is compared with another one whose complement is 4A.XXXY, one finds that the only difference is an extra Y which in fact has shifted the sex from complete femaleness to maleness. Another plant resulting from non-disjunction, has the complement 4A.XXXXY and is strongly hermaphroditic. It differs from the strong female 4A.XXXX in an extra Y-chromosome.

In other words, in a complement where one Y-chromosome is opposed by three X-chromosomes the resulting plant is still mainly male. This led Warmke to believe that the Y-chromosome must have strong male determining genes.

Warmke also found that the X-chromosome has female determining genes. This is because, if we take the X/Y ratio and consider plants forming a



series of increasing Xs, the result is progressively increasing femaleness. A plant with the complement 2A.XY is a strong male, but when the complement is 2A.XXY, the plant is still a male, but we get the rare hermaphrodite blossom. This extra X-chromosome can be arrived at through outbreeding and non-disjunction. The 4A.XXXY plants are predominantly males, but occasionally produce blossoms of a hermaphrodite nature. But in the 4A.XXXXY plants, such blossoms almost always result. Therefore from 2A.XY plants which are strictly males to the 4A.XXXXY plants which have equally well formed male and female sex organs, there is a tendency for increase in femaleness with increase in the number of the X-chromosomes opposed to the Y-chromosome.

The different ratios of sex chromosomes in Warmke's plants were arrived at through:

- (a) Selecting non-disjunction types which lack or have an excess of specific individual chromosomes.
- (b) Crossing triploids or tetraploids with diploids.
- (c) Crossing various polyploids from which euploid types with new ratios of sex chromosomes (XXY, XXXY) were produced through segregation and recombination.

The autosomes according to Warmke do not have an important part to play in sex determination in Silene. This conclusion was also arrived at by Blackburn (1923), Winge (1931), and Löve (1944).

Warmke found out that the opposing genes are in the homologous or partly homologous chromosomes (X and Y), and segregate to determine the two sexes. He then goes on to discuss the sex potentialities as distinct from sex determination. In Silene, the male determiners are in the Y-chromosome, while the female ones are in the X-chromosome. He states that this does not mean that females with only XX-chromosomes are completely females with no signs of any maleness. On the contrary, very young blossoms are



indistinguishable, and both have pistil and stamen primordia. In fact they are fundamentally bisexual; and any outside influence such as attack by Ustilago violacea would verify this. The smut would cause the rudimentary stamens in the female flower to develop into mature ones with the anthers filled with the fungus spores in place of the pollen. The female plants therefore have sex potentials for both femaleness and maleness, but have sex determiners for femaleness only. Sex determiners may well act to complete an essential step in the complex series of events in sex development. It is not surprising that female plants should have male characteristics inherent in them, and be able to show them when various conditions arise. Warmke states that, "...It is not inconceivable to see that the fungus may act as a partial substitute for the genes in the Y-chromosome in producing stimulations or substances that function as male sex determiners!"

Lindsay (1930) reviews dioecism in a number of Angiosperms, and states that the origin of the difference between the male and the female complements can be traced back to fertilization. He refers to sex in angiosperms as by no means being unalterable. In the case of Silene dioica with the XY set up, hermaphrodites are known to occur with the normal male complement. He arrives at the same conclusions regarding the shape and size of the sex chromosomes as described first by Blackburn (1923) and later on by Winge (1931), Ono (1939), and by Löve (1940-44).

Baker (1947) compared the action of Ustilago violacea (Pers.) Fuckel. on Silene to that of the animal hormones - testosterone and oesterone. He remarked that when such sex reversals do occur, some and not all the sexual characters are affected. The sex linked characters worked out by Shull (1914), Winge (1931) and Baker (1947), which are listed below are not affected by such reversals. These sex-linked characters are:



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1. "Angustifolia". This character described by Shull, (1914) is X-linked and lethal to pollen.
2. The shape and size of the calyx and its teeth as described by Baker (1947).
3. "Aurea" - Winge (1931) is an X-linked character and lethal to the plant when in the homozygous state. Aurea plants which are all males have a yellowish green colour, and are not evenly coloured. Aurea plants are weak and do not flower in the first year, and commonly die in the winter after flowering.
4. "Abnormal" - Winge (1931). This is a recessive character located in both the X and Y chromosomes. Here the upper part of the plant is paler in colour, and the calyx is never quite open even when the plants are in full flower.
5. "Variegated" - Winge (1931). Three autosomal genes, plus one located in the Y chromosome, inhibit this character. The result is that plants which do ~~not~~ show it are all females. Winge found, that out of a total of 2715 variegated plants, only 14 were males. Even then, the 14 plants were not completely variegated.

Winge considers that his experiments showed that the sex determining genes are present in the X and the Y chromosomes and that the occasional hermaphrodites owe their origin to a disturbed balance between genes pulling in male and female directions.

Baker (1947) postulates that in staminate plants, a substance akin to testosterone or male animal hormone is formed, but that this does not preclude the production of another one similar to oesterone in quantities insufficient to affect the morphology. He also argues that a concentration



of testosterone just enough to suppress pistil formation and promote that of stamens, might be produced under the influence of one set of genes, while an additional production of testosterone caused by another set of genes would induce the abortion of pistils. Thus the two sets of genes could have the same action and neither be specifically a female suppressor or male promotor. This view, according to Baker, is supported by the fact that, "....Purely pistillate plants when infected with the smut Ustilago violacea, produce well formed stamens, while the abortion of the pistil varies in extent and is never complete.

Westergaard (1958) in dealing with sex determination in dioecious flowering plants, refers to dioecism in Silene as being so unstable that bisexual forms in normally dioecious types are found in nature in rather high frequency, and are normally fertile.

Westergaard supports Blackburn in her description of the heteromorphic sex chromosomes in Silene. He worked on natural hermaphrodites, which when he examined cytologically, proved to be heterogametic or in fact, modified males.

Through a series of genetical experiments, Westergaard managed to map out the Y-chromosome, and came to the conclusion that the distal part must be containing genes suppressing the female expression, and part of the arm must contain genes which control the first stages in anther development, while the middle part of the Y-chromosome must include genes that control the initiation of anther formation. Thus the Y-chromosome determines sex through complete linkage with genes which suppress female development, and genes which initiate and complete anther formation.

An interesting point raised by Westergaard is that hermaphrodites in Silene always have a Y-chromosome or at least a fragment of one, - that is, an element of maleness. The only exception to this is the case when these hermaphrodites are induced by the fungus Ustilago violacea.



According to Westergaard, "...Evidently the fungus can do the same trick as the Y-chromosome - perhaps through the production of some hormones."

In the general evolution of sex Westergaard, postulates that an end product comes about through a series of steps, each controlled by a certain gene, and it can be short-circuited anywhere a gene is blocked. If M and F, be the end products for maleness and femaleness respectively, and going through simultaneously in ten steps -  $M_1 \rightarrow M_{10}$  and  $F_1 \rightarrow F_{10}$ , and if none of the genes is blocked, then the plant is a pure bisexual. But if  $M_5$  is blocked, then we have a female with staminodia, or if  $M_8$  is blocked, then we get a sterile male. All this depends on how far maleness must have gone before the blocking took place. This is in keeping with the normal physio-chemical reactions.

Löve (1940 and 1944) studied sex determination <sup>in</sup> Silene. This work supported the view that the male is the heterogametic sex, and that the Y-chromosome is the larger one of the two sex chromosomes. The point that she brought out here, and in which she agrees with Warmke, is that when the tetraploid individuals with the complements 4A.XXXX and 4A.XYYY are crossed, the offspring is a male. One of the sex chromosomes she found to be clearly larger than the other, and it was the Y-chromosome. Love came to the same conclusion that sex in Silene is entirely dependent on the X to the Y ratio, though in nature there are intersexes with the normal number of chromosomes, ( $2n = 24$ ). These intersexes are either gynohermaphrodites or androhermaphrodites, depending on whether they are predominantly females or males.

Löve collected some Silene plants and checked their sexes. One of the plants which was labelled as a pure male in the spring, produced intersexual flowers in the next summer. The reason given by Love for this phenomenon, is that the purely male branches developed in the cooler spring weather, with its long wet periods, while the ones bearing the intersexual flowers, appeared in the hot and dry periods. In Iceland, Love reports that intersexual plants are very frequently encountered at



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the end of July, when the temperature reaches 30°C. From this it may be inferred that sex determination is also influenced by environmental factors.

Heslop-Harrison (1957) though he does not specifically refer to Silene but to higher plants in general, supports the view that environment plays an important part in influencing sex determination in plants. He states that mineral nutrition, light, and temperature have direct influence on sex expression. A high concentration of nitrogen, for example, promotes femaleness and depresses maleness in long-day plants such as spinach. Light regimes seem to affect the plants production of auxins which in turn regulate the sex of the flower, since the optimum level of auxins for staminate expression is lower than for pistillate expression.

Hillman (1964) working on the physiology of flowering states that sex expression in plants is frequently influenced by factors of the environment. He cites light as being one of the more important of these environmental factors, and gives the example of the effect of light on the Acorn squash (Cucurbita pepo). This plant produces one flower primordium per node. But these primordia develop differently depending on the amount of light they receive. The primordia which are getting the full benefit of the light source, give rise to under-developed males. "Feminization" progresses in stages down the branch, until at the lowest nodes, the flowers are comparatively large and purely pistillate. This means that when the amount of light received by the primordia is diminished, there is a greater tendency for female flowers to be evolved, and male features to be suppressed. In this plant, therefore, light favours male expression.

Chemical control of sex has been studied in a number of plants. Löve (1945) showed that in Silene, animal hormones had some significant results. For her experiments, the animal male and female hormones - testosterone and oesterone were used. The top sprouts in Silene were cut off and either of the two hormones in the form of a thick paste was placed in the



axils of the uppermost leaves. Two weeks later on, hermaphrodite flowers appeared. The staminoidia in normal females developed into full stamens when treated with the male hormone - testosterone. Normal males have no traces of femaleness in them except for a tiny hair-like body in the middle. When these are treated with the female hormone - oesterone, a small pistil is formed.

With regard to sex ratios in Silene, Shull (1912) states that sex in Silene does not accord well with the theory which requires that in a dioecious species, males and females should be present in equal numbers. The ratios he found in his material was four females to three males. To this Love adds, that many workers (results listed below), have found that in Silene, there is a preponderance of females over males, as is the case with many dioecious plants.

This preponderance of females when it does occur is probably (according to Love) ascribed to the competition between male and female determining pollen grains. Two types of such grains are produced. In one type there is a Y-chromosome that is lacking in the other. The grains with the Y-chromosome are believed to be smaller in size, containing less nutriment, and, therefore, have less chances of withstanding the adverse conditions. But of course this theory does not stand well to the test, since in a number of localities, preponderance of males is noticeable. I have also found out, in my field work, that one cannot decide as to whether there should be more males or females since results from different localities differ.

## SOME RECORDS OF USTILAGO VIOLAE IN BRITAIN.

List of ratios drawn out by LöveProbably the first reference to the disease caused by *Ustilago*

|                                | <u>No. Males</u> | <u>No. Females</u> | <u>% Males</u> |
|--------------------------------|------------------|--------------------|----------------|
| Girou de Busareignes<br>(1831) | 692              | 522                | 54.6           |
| (1833)                         | 1072             | 1088               | 49.6           |
| Strasburger (1900)             | 142              | 173                | 45.0           |
| (1910.a)                       | 376              | 659                | 36.3           |
| (1910.b)                       | 77               | 107                | 41.8           |
| Correns (1917.a)               | 381              | 895                | 29.9           |
| (1917.b)                       | 555              | 737                | 42.9           |
| Åkerlund (1927)                | 30               | 57                 | 34.5           |
| Warmke and<br>Blakeslee (1939) | 20               | 17                 | 54.0           |
| Löve. D, (1940)                | 729              | 493                | 59.6           |

for appreciable amounts of money.

Baker (1947) mentions various localities in Great Britain, and Wales. In the Lower Peninsula of Germany, it is very common and in some parts very largely destructive.

Ainsworth and Spegarn (1955) include it in their list of British smut fungi. The disease is in fact very common in some parts of Britain where the host plants flourish.



SOME RECORDS OF USTILAGO VIOLACEA IN BRITAIN.

Probably the first reference to the disease caused by Ustilago violacea to British plants appeared in Sowerby's "British Fungi" where it is referred to as Farinaria stellariae.

Plowright (1889-1892) refers to this fungus as infecting the anthers of Lychnis diurna and Lychnis vespertina.

Dod (1889) found the disease very prevalent in <sup>the</sup> his woods in the South of England, on flowers of the Rose campion (Lychnis diurna).

Doncaster (1912) found the fungus very prevalent around Cambridge, infecting the flowers of "Lychnis" dioica and "Lychnis" vespertina.

Erikkson (1930) gives a list of British plants that are known to be attacked by this fungus. He adds the genus Vicaria to the list given by Plowright.

White (1936) lists a number of British plants belonging to the genera Cerastium, Silene, Saponaria, Stellaria, and Dianthus. He cites the variety on Dianthus caryophyllus as the only case where the disease has a real economic importance, since this host plant is grown under glass and marketed for appreciable amounts of money.

Baker (1947) mentions various localities mainly in North East England, and Wales. In the Gower Peninsula of Glamorgan, he found the Red campions very common and in some parts very highly infected.

Ainsworth and Sampson (1950) include this fungus in their list of British smut fungi. The disease is in fact very widely spread in all parts of Britain where the host plants flourish.

Violacea (Pers.) Pösch. In the Gower Peninsula, the Red campions were found infected, while in the fourth locality, the Red campions were more or less infected.

Two male plants and two female plants were taken from the infected plants, put in polythene bags and taken to the Department's glass house and in a green house for further observations.

From this area also, seeds were collected from mature capsules and stored for future treatments.

Diseased plants were transferred from Boarhills to the Department, and seeds from this heavy MATERIALS AND METHODS. also collected.

#### SOURCES OF PLANTS AND CHLAMYDOSPORES.

Silene dioica (L.) Clairv. is very prevalent in the country around St. Andrews. One finds this species flourishing especially in protected parts at the roadside. In all the localities I have seen, this species tends to grow on sloping ground, high cliffs, and under thickly wooded places that are also sloping, but never in open fields. It appears, therefore, that the natural habitat for this species is well drained soil with a fair amount of protection by larger vegetation.

Four localities in the neighbourhood of St. Andrews were selected for the collection of material and for field observations. These localities were:-

1. Roadside at Stravithie which is about 2 miles from St. Andrews.
2. Roadside towards Boarhills - 2 miles from St. Andrews.
3. Kinkell Braes which is a steeply sloping sea cliff just outside the residential area of St. Andrews.
4. Boarhills, a thickly wooded spot with ground sloping down to a small stream. About 4 miles away from St. Andrews.

In the first two localities, all the plants are free from Ustilago violacea (Pers.) Fuckel. In the third locality only one plant was found infected, while in the fourth locality, the majority of the plants were more or less infected.

Two male plants and two female plants were dug out from Stravithie, put in polythene bags and taken to the Department where they were kept in a green house for further observations.



From this area also, seeds were collected from mature capsules and stored for future treatments.

Diseased plants were transferred from Boarhills to the Department, and seeds from this heavily infected area were also collected.

Smut chlamydospores collected from Boarhills were obtained either by taking whole branches containing smutted flowers, putting them in polythene bags, or taking individual infected flowers and putting them in screw topped bottles. In both cases, storage was either under room temperature (18-19°C), or in a refrigerator kept approximately at 0°C.

#### FIELD OBSERVATIONS ON DISEASED PLANTS

The locality at Boarhills, where diseased plants were found in great numbers, is an isolated spot, being the site of an old flour mill generating its power from a small stream below.

The locality is dominated by some very tall trees. The Silene plants mixed with some other plants form a thick undergrowth. The steeply sloping nature of the ground is perhaps ideal for this plant, which apart from the incidence of the disease, seems to be the dominant plant in the vegetation underneath the trees.

Infected plants in this area were clearly more numerous than the healthy ones. Without examining the flowers, one could tell with some accuracy as to whether a plant was healthy or diseased, for in the infected plants, the branches were very profuse and weak, resulting in a more or less creeping habit. Also, though not always, the colour of the leaves was paler in the diseased than in the healthy plants.

Microscopic examinations of infected flowers will be discussed later.

During this course of study, many visits were made to the Boarhills locality. Two of these visits were devoted to the investigations on the percentages of naturally infected Silene dioica plants.

The first visit was made in the early summer (June 1963), while the other was in the autumn (September 1964). The plan was to find out the ratio of infected plants to healthy ones. Also to find the ratio of healthy males or healthy females growing amongst heavily infected plants.

In the first visit, 120 plants were counted. 92 of these plants were infected. Of the healthy ones, 24 were female and only 4 were male. Thus the percentages were:-

|                     |   |     |         |
|---------------------|---|-----|---------|
| Total No. of plants | = | 120 |         |
| " " " " infected    | = | 92  | = 76.7% |
| " " " " healthy     |   |     |         |
| females             | = | 24  | = 20%   |
| " " " " healthy     |   |     |         |
| males               | = | 4   | = 3.3%  |

In the second visit 80 plants were counted in the same area. Infected plants were 58. Healthy females were 17 and healthy males were 5.

The percentages were:-

Healthy females = 21.25%  
" males = 6.25%

The above results show that the male plants are more susceptible to the fungus than the female plants. It is also an indication that towards the end of the season, plants which were showing the disease earlier on, came out with healthy flowers, or that they have grown away from the disease.

### SEX RATIOS.

Löve (1940) reports that many workers (see introduction), have found that in Silene dioica there were more females than males in any one locality, though she, according to her own figures, found quite the opposite. But why should there be an excess of any one sex at all? It would be expected that the sexes should be equally represented.



With this question in mind, field observations were carried out in the first of the three areas previously mentioned, namely Stravithie, the roadside towards Boarhills, and Kinkell braes. Boarhills was excluded because of the great incidence of the Smut.

Stravithie. The plants in this locality grow parallel to a drainage ditch, and between it and a farm hedge. A stretch of about 80 yards of this was examined. The total number of plants counted was 74 of which 32 were female and 42 were male. Care had to be taken here, because of the creeping habit of the branches, one might by mistake have counted one plant twice.

Roadside to Boarhills. In this locality, S. dioica plants grow under very similar conditions to those in the first locality. Here 62 plants were counted, of which 28 were female and 34 male.

Kinkell braes. The plants here are much larger and cover a wider patch of land. The number of plants counted was 94, with 49 female and 45 male.

Taking the three areas together, the total number of plants recorded was 230, with 108 female and 122 male. Therefore the female plants formed 46.6% and the males 53.4% of the total number of plants recorded in the three chosen areas.

The figures of male and female percentages quoted here show a small departure from the theoretical ratio of 1:1. This might be explained by the fact that the total number of plants taken was not large enough.

The significance of this data can be assessed by  $\chi^2$  test (Loomis and Shull (1937)). The  $\chi^2$  value deduced for this data, assuming a normal 1:1 sex ratio, is 0.85. This result may be taken to indicate that the true sex ratio for S. dioica is in fact 1:1, and this was further confirmed in later experiments with root-tip squashes where results obtained were closer to the theoretical ratio.



Apart from this field work, some plants, which were grown from seed, were kept in the green house and brought forward to flowering. These plants were either raised specifically to determine the sex ratios, or they were used as control in inoculation experiments on seedlings and adult plants. The total number of green house plants which were raised at the various seasons and which survived till flowering was 84. Of these 34 were female and 50 were male, giving the percentages 40.5 and 59.5% respectively.

#### ROOT-TIP SQUASHES

Squash methods for root tips were employed with the object of establishing the sex ratio by chromosome examination. For these experiments, actively growing roots were obtained by germinating seeds on wet filter paper and using the radicles as material for the two cytological methods mentioned below.

Seeds were first collected either from the localities mentioned previously, or from the green house and kept in the laboratory where the temperature was kept steadily at 18-19°C.

Two fixing and staining methods were employed:

##### 1. The Feulgen Method

The roots were placed in aqueous solutions of colchicine (0.025%) for about four hours. This ensures larger proportions of cells at metaphase, during which chromosomes can be counted and studied with greater ease. The roots were then fixed in acetic acid/ethyl alcohol fixative formed of three parts absolute alcohol and one part glacial acetic acid and a trace of formalin. After being in the fixative for 24 hours, the roots were removed and washed thoroughly in distilled water. This was done by leaving them in distilled water in specimen tubes and changing the water at half hourly intervals. Four changes of water were found to be necessary to remove all traces of the fixative, especially as it contained formalin.



The roots were then hydrolysed for 8-10 minutes in N.HCl using a thermostatically controlled water bath at 60°C. The roots were washed in distilled water and then left in the Feulgen stain for two hours.

A slide was then cleaned in absolute alcohol and the root apex containing the apical meristem was tapped out in a drop of 45% acetic acid. A number "0" cover slip was made sticky with one drop of glycerine albumen smeared over it, warmed a little over a flame to make it adhesive, and then placed over the root apex on the slide. The cover slip was held tightly in position, tapped with a needle to separate the cells, and then squashed.

The slide was then inverted over a staining dish containing 45% acetic acid, and left there until the cover slip floated away. The cover slip was rinsed in 95% alcohol for two minutes followed by another two minutes in absolute alcohol and mounted in Euparal.

The Feulgen stain was prepared according to the schedule given in Darlington's "The Handling of Chromosomes" (1960).

The stain was kept in a tightly-stoppered dark bottle stored in a refrigerator at 0°C.

## 2. Cresyl violet

When dealing with large numbers of root tips, where the preparations were not destined to be photographed, then quicker methods of staining the chromosomes were found useful. The schedule first proposed by Stewart and Schertiger (1950) and modified by Amerkhanian (1964), was adopted. The modified stain was 1% Cresyl violet in 50% acetic acid.

The roots were first fixed in ethyl alcohol/acetic acid fixative for 10-15 minutes, transferred to acid alcohol (one part concentrated HCl to one part 95% alcohol), kept there for up to three minutes, washed in 70% alcohol and left in the stain for one to two minutes. The roots were then washed in tap water and squashed under number "0" cover slips, and observed as temporary preparations.

The chromosomes showed very well in these preparations, although the background was not absolutely clear, but the shape and size of the

individual chromosomes were easily seen especially the sex chromosomes which are clearly distinct from the rest of the autosomes and from one another.

By employing methods "one" and "two", the sex ratios from root tips squashes were obtained. Either seeds were taken from the stored stock in which any number were germinated, and the males and females were calculated as percentages of that number or all the seeds from a single capsule were germinated (usually in two lots since it was found that the seeds that came out from any one capsule were too many to be treated all at the same time) and the percentages of the total calculated.

Before determining the sex ratios, experiments were conducted to establish the male and the female complements and the shape and size of the sex chromosomes.

(1). Root tips from known male or female plants were taken out and the Feulgen method was employed in examining the chromosomes and comparing the two complements.

(2). Anthers and ovaries were treated either in the Feulgen method or the "Propionic orcein" stain. In the latter case, the schedule recommended by Warmke and Blakeslee (1940) was found very suitable. In their formula, however, they used carmine which was substituted here by orcein because of the clearly superior results obtained. The stain was prepared by adding 1 gm. orcein to 99 c.c. of 45% propionic acid. The mixture was boiled over a low flame for about 30 minutes, cooled to 50°C and filtered.

From the male plant, buds containing dividing cells were found to be 1.5-2.5 mm. in length. Such buds were fixed in 3:1 absolute alcohol/acetic acid (glacial) in which they were left over night. The next morning, the buds were removed from the fixative, washed in three changes of distilled water, and transferred into the stain where they were left for another 24 hours.

Anthers were then crushed out in fresh drops of the stain on a slide,



covered with cover slips, squashed and used as temporary preparations. When permanent preparations were required, the cover slips were floated away in the same manner as in the Feulgen technique.

The same procedures were employed in the case of buds from female flowers. The buds used here were 2-3 mm. long.

The above investigations confirmed that the chromosome complement of both male and female plants is 24. The shape and size of the X and the Y chromosomes coincide with the descriptions referred to earlier.

#### Results of Root tip Squashes

##### A. Seeds from stock.

| <u>Total number</u> | <u>Males</u> | <u>Females</u> | <u>%age males</u> | <u>%age females</u> |
|---------------------|--------------|----------------|-------------------|---------------------|
| 76                  | 36           | 40             | 47.37             | 52.63               |
| 58                  | 30           | 28             | 53.45             | 46.55               |
| 35                  | 19           | 16             | 57.14             | 42.86               |
| 46                  | 22           | 24             | 47.82             | 52.18               |
| <u>94</u>           | <u>51</u>    | <u>43</u>      | <u>53.12</u>      | <u>46.88</u>        |
| 309                 | 158          | 151            | 51.13             | 48.87               |

##### B. Seeds taken from single capsules.

| <u>Total number</u> | <u>Males</u> | <u>Females</u> | <u>%age males</u> | <u>%age females</u> |
|---------------------|--------------|----------------|-------------------|---------------------|
| 108                 | 56           | 52             | 51.85             | 48.15               |
| 116                 | 54           | 62             | 46.55             | 53.45               |
| 128                 | 58           | 70             | 45.32             | 54.68               |
| 176                 | 94           | 82             | 53.40             | 46.60               |
| 144                 | 70           | 74             | 48.54             | 51.46               |
| <u>138</u>          | <u>76</u>    | <u>62</u>      | <u>55.07</u>      | <u>44.93</u>        |
| 810                 | 408          | 402            | 50.37             | 49.63               |

It appears from the above two tables, especially "B" in which all the seeds in each of the six experiments were taken from one capsule, that there is no reason to justify the assumption that there are in

S. dioica more plants of one sex than there are of the other. Considering the table given by Löve on page 24, one notices that when very large numbers of plants were examined, the percentages of male and female was very nearly 50 or the ratio was 1:1. The figures of Girou De Busareigues in that table are a good example of that. One would assume that <sup>in</sup> any one locality, the male and female plants should be evenly distributed, since segregation has its origin at fertilization. The zygote has an equal chance of developing into an embryo of a potentially male or female plant.

The  $\chi^2$  value deduced for the data in the table assuming equality for the sex ratios is 0.044. When compared with the  $\chi^2$  value deduced for the data on field observations given on page 29, it is noticed that the latter data are of lesser significance to the establishment of the hypothesis of the equality of the sex ratios.

The  $\chi^2$  value for this data is 0.044 which is much smaller than the calculated value (Loomis and Shull), establishes the hypothesis that the two sexes are evenly distributed in any one locality.

### INOCULATION STUDIES

#### 1. Seed Transmission.

Baker (1947) reports that ~~§~~Brefeld (1883)~~‡~~ advocated the theory of seed transmission of the disease caused by U. violacea. In some of the smuts, particularly those attacking cereal plants and where the spore balls are formed in the ovaries, infected seeds are in fact the main vehicles through which the various diseases are transmitted to the next generation. But does this apply to Ustilago violacea, where the spores are not formed in the ovaries? If it does, then it would imply at least, that, fully developed seeds can be obtained from infected female plants. This I have not managed to observe, since in all the infected flowers examined, those ovaries which developed and which possessed ovules, never formed seeds, either viable or abortive.

However, three experiments were performed as follows, to prove that the disease was not seed transmitted.

A. Seeds were collected from female plants growing in the heavily infected



area at Boarhills. A preliminary check was carried out to detect any spores adhering to the seed coats. To do this, a seed was crushed on a glass slide, bits of the seed coat were collected by a fine forceps leaving the rest of the debris. Very small bits of the seed coat were placed on another slide in a drop of cotton blue in lactophenol and left to soak for some time, being rocked frequently with a fine needle. After placing a cover slip on that preparation, microscopic examinations of the seed coat and the liquid around it did not reveal any spores. This was repeated many times but always with negative results.

Next, some seeds from the same area were germinated on filter paper and later 15 of the seedlings were transferred to pots and kept in the green house. When these plants flowered, all the flowers were healthy.

This shows not only that spores are not carried by the seeds but also that there are no dormant mycelia in them.

B. Since U. violacea has been shown to have physiologic specialization (Goldschmidt, 1928), the spores to be used in inoculation experiments had to come from smutted flowers of the same species.

Some seeds were shaken in a closed bottle containing a large number of chlamydospores, thus ensuring that the spores adhered to the seeds. The seeds were sown in small pots to germinate and the seedlings were transferred to larger pots where they were maintained till flowering. All the 16 plants thus raised showed no signs of infection.

C. Seeds soaked for three hours in water containing a thick suspension of chlamydospores, were germinated and were treated as in "B".

Of the 16 plants raised and flowered, only one showed partial infection that is infected flowers were seen in one branch only. If the infection in this case was due to spores adhering to the seed coat rather than to spores infecting the emerging seedling, then seed infection which was much more rigorously attained in these experiments, than could occur in nature, was responsible for only a low percentage of infections.

It is suggested from the above results, that seed infection cannot



be the natural method of conveying the disease from one Silene plant to another.

## 2. Flower infection.

Reference has been made earlier to the workers who have noticed insects visiting both healthy and infected flowers. On the many visits I have made to Boarhills, the locality where S. dioica was heavily infected, I also noticed this. A strikingly large number of small flies did hover around the plants. I have trapped numbers of them inside some of the healthy and the diseased plants. Obviously these flies were collecting pollen or nectar, but they did carry both spores and pollen grains on their bodies. There could be no doubt that some of the spores taken from an infected flower would be deposited on a healthy one. The question arises now as to whether infection does occur after the deposition of the spores on the flower. Plowright (1892) writes that he succeeded in infecting a healthy female plant by applying some chlamydospores on the stigmata. The following experiments were aimed at clarifying this.

Spores were placed in small vials containing distilled water and left there for two to three hours before the suspension was drawn into a very fine pipette. The liquid containing the spores, hereafter referred to as inoculum, was injected into the open male and female flowers. It was necessary to make sure that no other part of the plant became contaminated. To avoid this, the basal part of the flower was wrapped with filter paper which sucked away any water trickling out. In each plant, about eight to 10 flowers were inoculated in this manner, making sure that the inoculated flowers were on the same branch. Eight male and eight female plants were treated in this manner.

## Results and Conclusions:

|             | <u>Number of plants inoculated</u> | <u>Number infected</u> | <u>Percentage</u> |
|-------------|------------------------------------|------------------------|-------------------|
| (i) Male    | 8                                  | 6                      | 75                |
| (ii) Female | 8                                  | 1                      | 12.5              |

In all the cases, infected flowers appeared after 5-7 months. As expected, these infected flowers were only in the branches where inoculations had been carried out. However, after another eight months,



other branches were noticed bearing infected flowers. (These plants were kept in the greenhouse, where the temperature was about 65°F, and with artificial light maintained for 14 hours a day. Therefore they produced flowers all the year round).

It is worth noting here that although both groups of plants were subjected to the same treatment and were kept under the same conditions, the results were markedly different. The female plants seemed to be less susceptible to flower infection than the male. But on the whole, one can say with some confidence that the flower is one of the main channels through which infection of S. dioica by U. violacea takes place.

### 3. Bud infection.

Since Hecke (1907) successfully infected plants of Silene alba - the white campion, by inoculating the buds in the axils of leaves with chlamydospores of U. violacea under humid conditions, experiments were performed to confirm that this mode of infection is also true for S. dioica.

A spore suspension was prepared as in (2) above. The buds inoculated in these experiments included terminal as well as axillary buds. The inoculations were carried out in one of two ways, and eight male and eight female plants raised experimentally and known to be uninfected were used in each case with ten buds in each plant.

(a) The buds, whether axillary or terminal, were covered with a thick suspension of chlamydospores using a fine brush. In every case the potted plant was given a good watering, placed in a deep tray containing some water and the whole plant was placed under a bell jar. This provided very humid conditions ensuring that the spores did not dry up before germination. The plants were left for at least ten days before the bell jars were removed.

### Results:

First signs of infections were observed from four to ten months after the inoculations.

| <u>Plants inoculated</u> | <u>Plants infected</u> | <u>Percentage infection</u> |
|--------------------------|------------------------|-----------------------------|
| (i) Male 8               | 5                      | 67.5                        |
| (ii) Female 8            | 2                      | 25                          |

(b) The other method employed in bud infections was to use a syringe and to inject the spores right into young buds. Again eight plants of each sex were inoculated and other precautions were the same as in (a).

#### Results:

| <u>Plants inoculated</u> | <u>Plants infected</u> | <u>Percentage infection</u> |
|--------------------------|------------------------|-----------------------------|
| (i) Male 8               | 6                      | 75                          |
| (ii) Female 8            | 1                      | 12.5                        |

Bud infection is therefore effective, and is perhaps more so in nature than flower infection, since one can suggest a greater chance that spores get caught in the axils of leaves rather than inside the flowers. Also there is a better chance for these spores to germinate in the water trapped in the axils of leaves, whereas such water is very rarely found inside flowers except after a very heavy rainfall.

#### Other methods of infecting adult plants.

##### 1. Inoculation of Stem and Leaf.

Doncaster (1912) tried, with little success, to inoculate male and female plants by rubbing chlamydospores on the epidermis of Lychnis (Silene) dioica. It was decided to repeat his experiments with some modifications.

All flowering shoots were removed from eight healthy male and female plants, to avoid the possibility of flower infection. Fresh spores were placed in glass vials and allowed to soak for about three hours. Spores were then taken up in a hypodermic syringe. Four male and four female plants were injected under the epidermis in one of the youngest of the remaining leaves, and a similar number were injected under the epidermis



in the stem, two nodes away from the growing point. These plants were kept under very humid conditions for five days before they were transferred to the greenhouse.

### Results and conclusions.

After seven months all the plants were again in full flower. Only one plant (a male inoculated under the epidermis of the stem) had infected flowers in one of its branches. After another five months more branches in this same plant were seen bearing infected flowers. All the other fifteen plants continued to produce healthy male or female flowers.

It is worth noting here that the first infected flowers looked very like ordinary healthy male flowers except for the spores, whereas infected flowers that appeared later were much thicker around the calyx, and gave the appearance of female flowers. The other point observed in one of the first infected flowers was that two of the anthers were full of pollen grains instead of smut spores. There were, however, more instances of this which will be discussed later (page 76).

The plants just mentioned were subjected to treatment that can hardly be expected in nature, yet infection can by no means be regarded as successful when using this method. It would therefore be reasonable to exclude infection from spores falling on leaves or being caught amongst the hairs on the stem as an important natural means of disease transmission.

### 2. Infection from spore infested soil.

Soil samples from underneath plants at Boarhills, were examined for U. violacea spores as follows:-

10 c.c. of distilled water was added to the soil sample, the grit was allowed to settle and a drop of the clear liquid was put on a clean slide. Many spores were seen under the microscope and many of them actually germinating. This prompted an attempt to cause infection through spores falling on the soil, germinating there, and attacking any available seedlings.

Some seeds were germinated on moist filter paper. Twelve of the resulting seedlings were placed in small pots containing soil to which a heavy infestation of spores had been added.

After 14 months all the plants from these seedlings were in full bloom. Only one plant produced infected flowers and then died shortly afterwards. This very low percentage suggested that the single plant had become infected through spores landing on the terminal bud in the seedling stage, through splashing with spores from the soil when watering, and that direct soil to seedling infection does not occur in nature.

#### Seedling infection.

Plowright (1892) wrote that he had tried to infect very young seedlings, but without success. Only then had he tried the flower infection mentioned earlier. Seedling infection is quite common with a number of those smuts which attack plants belonging to the family Gramineae. Gäumann (1950) cited U. violacea as one of the smuts where infection of the host plant is normally through the seedling, although he does not exclude shoot infection in the adult plants.

Seedlings occur freely in nature growing under or close to mature plants in flower. Some experiments were performed to investigate seedling infection in S. dioica.

A large number of seeds were soaked in water for about three hours and then transferred to moist filter paper and allowed to germinate. After a week, the seedlings were about an inch tall, and their first foliage leaves were visibly forming in the terminal buds. Such seedlings were then treated in one of the two ways given below.

(a) Freshly collected chlamydospores, were placed together with some sterile water in small petri-dishes. Some of the seedlings were placed in these petri-dishes which were then covered. The seedlings were allowed to soak in this water with the heavy suspension of spores for about six hours before they were removed.



Thirty six seedlings were transplanted into small pots.

#### Results:

When the above plants came into flower in about ten months, of the original thirty six seedlings, 30 developed into infected plants, four were healthy females and two were healthy males.

Later on, when more branches came out of the infected plants, it was found they all were carrying infected flowers. A comparison should be drawn here with infections resulting from inoculating adult plants where infected flowers were seen in only some and not all branches.

These results have been summarised in table below:-

|                               |   |    |         |
|-------------------------------|---|----|---------|
| Total number of plants raised | = | 36 |         |
| " " " " infected              | = | 30 | = 83.3% |
| " " " healthy females         | = | 4  | = 11.1% |
| " " " males                   | = | 2  | = 5.6%  |

(b) The other method for inoculating seedlings, was by injecting chlamydospores into the terminal buds using a syringe. Each seedling after such a treatment was held upright in a stoppered specimen tube containing a little water into which the root system dipped. This ensured a very humid atmosphere suited for infection.

After six hours, the seedlings were taken out and potted. Only twelve seedlings were used.

#### Results:

Of the twelve plants raised, seven came out with infected flowers. Two were healthy females, one was a healthy male, and two plants died before flowering. If we take ten as the number of plants used, then the results could be summarised as:-

|                               |   |    |       |
|-------------------------------|---|----|-------|
| Total number of plants raised | = | 10 |       |
| " " " infected plants         | = | 7  | = 70% |
| " " " healthy females         | = | 2  | = 20% |
| " " " " males                 | = | 1  | = 10% |

When we add (a) and (b) we get the following results for seedling infection:-

|                               |   |    |          |
|-------------------------------|---|----|----------|
| Total number of plants raised | = | 46 |          |
| " " " infected plants         | = | 37 | = 80.44% |
| " " " healthy females         | = | 6  | = 13.04% |
| " " " " males                 | = | 2  | = 6.52%  |

The tables in this page and the previous one do not give the sexes of the infected plants, because this could not be accurately ascertained without examining the chromosome complements.

It appears from the above results that seedling infection is undoubtedly a very effective method of conveying the disease due to U. violacea. This perhaps explains why one frequently finds that infected plants form definite patches in a population where most of the plants are free of the disease. What may have happened here was that spores fell from infected flowers and became trapped in the terminal buds of seedlings growing underneath. The seedlings are so covered by the large plants that the atmosphere around them is usually humid and so ideal for infection.

Distilled water was found to be as good as tap water for the study of germinating chlamydozooids.

A drop of water was placed at the centre of a sterile petri-dish and spread out to cover a circle of about 1 cm. in diameter. Spores were sprinkled on the surface of the water. A petri-dish was prepared with a filter paper saturated with water. The whole set-up was transferred into the petri-dish which was then covered. The whole set-up was incubated at 22°C (later it was found that room temperature 18-20°C was more



SPORE GERMINATION

Ustilago violacea (Pers.) Fuckel. forms its chlamydospores in the anthers of its host plant - Silene dioica (L.) Clairv. In the cases investigated, the spore masses are powdery when they are ripe and have a pinkish brown colour. A single spore, however, shows only a very pale violet colour. Under the microscope and without using any stain, two distinct walls are visible. The inner wall - the entin - is very thin and colourless, while the outer wall - the exin - is thicker and highly echinulate giving the whole spore a rough surface.

The chlamydospores of this species are almost spherical, ranging in size from five to twelve microns with an average diameter of eight microns. When the spores were stained in cotton blue in lactophenol, it became possible to measure the reticulations on the exin, since those did not take the stain as well as the rest of the spore. These reticulations on the average measured  $1\mu$ . These measurements were made by employing a combination of a stage micrometer and an eye-piece micrometer allowing readings correct to  $1\mu$ .

Germination in Water.

It was found that the chlamydospores of this species had no rest period. When fresh, they germinated after only five hours' immersion in water. Some spores taken from unopened buds were found to germinate in about the same time as those taken from open flowers, thus showing that spores, are fully developed before the flowers open. In fact infected anthers normally dehisce long before the buds open.

Distilled water was found to be as good as any other medium for the study of germinating chlamydospores.

A drop of water was placed at the centre of a clean slide and spread out to cover a circle of about 1 cm. in diameter. Fresh spores were sprinkled on the surface of the water. A petri-dish was prepared with a filter paper saturated with water. The slide was ~~then~~ transferred into the petri-dish which was then covered. The whole set-up was incubated at  $22^{\circ}\text{C}$  (later it was found that room temperature  $18-20^{\circ}\text{C}$  was more



convenient for these experiments).

After six hours, the spores were stained with cotton-blue, warmed for about 30 seconds, covered with a cover glass and examined.

### Results

It became quite clear that germination must have started long before the slides were examined, since nearly all the spores had already germinated. Therefore the experiments were repeated with progressively shorter periods of incubation. The critical time for germination at 18-20°C was found to be four hours and fifteen minutes. This minimum time was confirmed by many observations.

A number of slides were thus made available giving a fairly wide range of possible stages in germination.

At the onset of germination, it was noted that the ~~exin~~ was pushed out slightly forming a bulge through which a germ tube came out. In the preparations showing the earliest stages, this tube was formed of a single elongated cell. Later on as this tube, or promycelium, increased in length, septa were laid down thus dividing it into, usually, three cells. Cytological studies on these promycelia revealed that the three cells were uninucleated. When the promycelium attained its full length, ca. 14 $\mu$ , it normally got detached from the spore. Usually another promycelium came out from the spore, either from the same point where the first one originated or from another point. It happened many times, that I have observed two promycelia either coming out from the same point in the spore thus forming a v-shaped structure, or from two different points (Fig. I & II Plate VI). In most cases one of the two promycelia was slightly larger than the other one. In the great majority of cases, these promycelia were three celled and very rarely one notices a promycelium with four cells.

When these promycelia became detached from the spores, they were spindle shaped with the two outer cells pointed whereas the central one looked very much like an epidermal cell in higher plants (Fig. II Plate VII).



Just before being detached from the spore, a promycelium normally budded off small conidia from its three cells. Apparently each promycelial cell budded off one conidium/<sup>or</sup>sporidium (De Bary), and that was the end of the part it played in the life history, because I have not observed any sporidia coming off promycelia after the latter became detached from the chlamydospores.

The sporidia when left in distilled water budded off secondary sporidia and the process was repeated for a maximum of four days, after which it was noticed that they started joining in pairs, having stopped this budding or vegetative growth.

#### Germination in Nutrient Media.

A few solid nutrient media recommended for the growth of smuts were tried. Some of these media were used with agar as the solidifying ingredient while in others gelatine was used. The references for these media are given below:-

1. Ranker's Medium developed for Ustilago maydis  
Jour. Agr. Res. 41. pp. 435.....1932.
2. Sartori's "Best" Medium developed for Tilletia caries  
Am. Jour. Bot. 11. pp. 617.....1924.
3. Haskin's MB-50 Formula (1950) in Fischer (1957)
4. Malt. Agar - 2% Difco - bacto agar. 2.5 malt extract.
5. Czapeks medium as modified by Dox (1910) in Smith (1960).

\*1 and 2 are gelatine media.

After each one of the media was prepared, some of it was poured into sterile petri-dishes and autoclaved for 20 minutes at 15 lb/square inch pressure. When the petri-dishes had cooled down, they were taken into the culturing room and the agar or gelatine was allowed to solidify.

Freshly collected spores were then used in inoculating each petri-dish at the centre. The petri-dishes were then stored in a culture store at



22°C. those extracted from ordinary yeast plants.

Of the above media used, three had agar and two had gelatine as the solidifying ingredients. Four petri-dishes were used for each medium. Germination of the spores was observed <sup>after</sup> 24 hours to have taken place in all the media. The mycelium which in all cases was in the form of yeast-like colonies grew from the centre towards the periphery. During the first two weeks, the culture in all the petri-dishes had a creamy-white colour with a very corrugated surface, which became darker as it aged. After two months the whole culture became pinkish in colour and its surface became hard and brittle in contrast with the interior which remained watery.

In the case of the gelatine cultures, when the fungus had developed for two weeks, the medium was hydrolysed, probably due to the activity of the fungus, with the result that the culture, which had been building up into a convex shaped body, started running to the sides. The whole topography of the culture was distorted. The same thing happened when the medium was made harder by increasing the percentage of the gelatine to 25%.

#### Growth Rate of Mycelium.

With the agar media, the culture grew steadily towards the sides of the petri-dishes forming a central circle increasing in diameter as it aged. This made it possible to measure the rate of growth of the fungus in two petri-dishes containing two different agars. The rate of growth was measured as increase in diameter per unit time. The diameter was measured in millimeters and the time in days. Readings were taken every three days for 30 days. After that time there was no appreciable growth, probably due to the drying up of the medium. A graph (Fig. I Plate I) was plotted showing this relation.

It is worth noting that in all the five media used the mycelium was in the form of yeast-like sporidia. An examination of the culture under the microscope revealed that these yeast-like cells were either solitary or formed long chains of budding sporidia which looked very much



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like those expected from ordinary yeast plants.

It was not possible to persuade the mycelial cells to develop into hyphae or aerial mycelia in any of the media used. Liquid media were then resorted to, using two of the above nutrient media without a solidifying ingredient. The results with liquid media were similar to those in the solid except for the colour and texture of the mycelium. In liquid media, the budding sporidia formed white gelatinous masses on the surface but never turned the pinkish colour seen with the solid media.

#### Effect of Temperature on Germination.

The relation of climatic factors, especially temperature to the development of smuts is a matter of great importance. Plant pathologists attach great importance to finding the cardinal points of temperature for the spore germination of any pathogenic smut, since such knowledge would help in assessing the dangerous times in the year when various plants are most susceptible to any such smut or indeed any fungal attacks.

Experiments were performed to find the effect of temperature on the germination of the chlamydospores of U. violacea. The medium in all these experiments was sterile distilled water.

Fresh spores were taken from infected flowers that had just opened. They were placed in a drop of sterile distilled water on a clean slide. The slide was then placed in a sterile petri-dish containing a disc of moist filter paper. At each temperature four petri-dishes were employed.

The dishes with their contents were transferred into a refrigerator specially fitted with a heater and a cooler and where the temperature is thermostatically controlled. With the cooler on, it was possible to register temperatures below  $0^{\circ}\text{C}$ , whereas for higher temperatures the cooler was not required.

It was found that in many cases, this thermostat did not hold the temperature steadily and within satisfactory limits. Since the spores



were not incubated for long periods, a mean temperature was recorded for each experiment (in plotting graphs), although the germination was considered to occur in the temperature interval from the maximum to the minimum temperatures. This difficulty in maintaining a steady temperature was greater at higher temperatures. Therefore all experiments with temperatures over 23°C were repeated in an ordinary incubator.

The slides were taken out and the spores fixed in absolute alcohol immediately after the expiry of the incubation period. This was necessary in order to stop spores from further germination. Preliminary experiments showed that the best duration for spore incubation was 16 hours.

Tables Showing the Rate of Germination  
of Chlamydo spores at Different Temperatures

| Temp.<br>°C | <u>A</u>                  | <u>B</u> | <u>B/A</u><br>% | <u>A</u>             | <u>B</u> | <u>B/A</u><br>% |
|-------------|---------------------------|----------|-----------------|----------------------|----------|-----------------|
|             | <u>In distilled water</u> |          |                 | <u>In 1% Glucose</u> |          |                 |
| Below 5     | 121                       | 2        | 2               | 103                  | 0        | 0               |
| 5-8         | 111                       | 46       | 41              | 93                   | 39       | 42              |
| 9-13        | 86                        | 59       | 70              | 83                   | 55       | 66              |
| 14-18       | 100                       | 111      | 100             | 104                  | 110      | 100             |
| 19-23       | 97                        | 110      | 100             | 99                   | 116      | 100             |
| 24-29       | 96                        | 77       | 81              | 118                  | 88       | 74              |
| 30-34       | 101                       | 40       | 40              | 104                  | 39       | 37              |
| 35-38       | 99                        | 4        | 4               | 97                   | 2        | 2               |

Column A - Total number of spores  
in field.

Column B - Total number of promy-  
celia in field.

The spores were then mounted in cotton blue and observed as temporary preparations.

Experiments of this type were performed at temperatures as low as 2°C

and as high as 40°C. There was very little germination below 5°C and none above 38°C. These two temperatures are therefore the two cardinal points or lowest and highest germination temperatures for the spores of U. violacea.

The percentage germination was arbitrarily taken as the ratio between the promycelia developed and spores in the field. This was necessary, because, as was previously mentioned, promycelia when they attain a certain size are separated off from the spore, in which case it becomes difficult to

Tables Showing the Rate of Germination  
of Chlamydo spores at Different Temperatures

| Temp.<br>°C | In 2% Glucose |     |          | In 5% Glucose |     |          |
|-------------|---------------|-----|----------|---------------|-----|----------|
|             | A             | B   | B/A<br>% | A             | B   | B/A<br>% |
| Below 5     | 94            | 0   | 0        | 103           | 1   | 1        |
| 5-8         | 103           | 42  | 41       | 93            | 42  | 45       |
| 9-13        | 101           | 55  | 55       | 110           | 82  | 75       |
| 14-18       | 91            | 102 | 100      | 95            | 116 | 100      |
| 19-23       | 97            | 115 | 100      | 104           | 127 | 100      |
| 24-29       | 96            | 77  | 80       | 100           | 76  | 76       |
| 30-34       | 97            | 30  | 31       | 91            | 46  | 51       |
| 35-38       | 86            | 1   | 1        | 102           | 1   | 1        |

Column A = Total number of spores  
in field.

Column B = Total number of promy-  
celia in field.

observe whether a certain spore has germinated and its germ-tube or promycelium has become detached, or whether it has not germinated at all. Of course when we count the promycelia, we are up against another difficulty arising from the fact that two or more promycelia counted may have come from the same spore, which would suggest a higher rate of



germination than has actually happened. But this is unimportant in this case, since we are merely comparing the results with other results under identical conditions, except for a difference in temperature.

It was not possible using the incubator to get temperatures at intervals of one degree centigrade. It was, however, possible to get temperatures at intervals of four to five degrees ranging from  $5^{\circ}\text{C}$  to  $38^{\circ}\text{C}$  and that <sup>was</sup> quite adequate for the experiments it was needed for.

The procedure described above for the germination of spores in distilled water was also followed when using dilute solutions of malt extract and glucose. The concentrations used were 1%, 2%, and 5%. There was no marked difference among these media or between them and distilled water as regards the cardinal points i.e.  $5^{\circ}\text{C}$  being the minimum and  $38^{\circ}\text{C}$  the maximum, nor in the optimum temperature of  $18-22^{\circ}\text{C}$  i.e. the range where there was 100% germination after 16 hours incubation.

Graphs were plotted to show the relation between temperature and percentage germination in the various liquids used.

An interesting point is that at the lower temperatures of 6 to  $11^{\circ}\text{C}$  all the promycelia remained attached to the spores, whereas at the higher temperature range of 26 to  $32^{\circ}\text{C}$ , at which almost equal rates of germinations occurred, the promycelia were detached immediately after the formation of their third cells. The same phenomenon was observed in all the cases where the different germinating media were used.

#### Age and Storage Conditions of Spores

It was mentioned previously that chlamydospores of U. violacea have no rest or after ripening period, but given the appropriate conditions, they germinated immediately after, or even before, the flower which contained them had opened. With regard to the germinating media, these spores apparently did not require any special stimulants for germination.

Experiments were performed to investigate the effect of age and storage conditions on the rate of germination. For this purpose a large



number of branches bearing infected flowers were taken from plants growing at Boarhills and transferred to the Department. In about half the sample, the flowers were opened and the spores scraped out into two bottles which were then only loosely stoppered. One bottle was stored at room temperature ( $18-20^{\circ}\text{C}$ ), while the other bottle was stored at  $0^{\circ}\text{C}$ . The remainder of the branches with the infected flowers were divided into two lots and each lot placed in a polythene bag. One lot was stored at room temperature while the other lot was stored at  $0^{\circ}\text{C}$ .

After 6 months, spores from all four sources were germinated in the way described earlier. All germinations were performed at the optimum temperature deduced from the previous experiments i.e.  $20^{\circ}\text{C}$  and incubations were for 8 hours and then repeated at 16 hours.

Similar examinations of the stored spores were made after 12, 18 and 24 months.

#### Results:

It appears from the two tables shown here that storage in whatever form it takes, has a direct effect on the viability and rate of germination of spores. Plowright (1889) suggested that the germinative faculty of the spores lasted only five months. This, however, is not the conclusion to be drawn from our experiments. Twenty four months was the longest storage period quoted here, and even then, there was a high rate of germination. The gradual decrease in viability of the spores with age is clearly marked by the steady fall in the percentage of germination ~~(The gradual decrease in viability of the spores with age is clearly marked by the steady fall in the percentage of germination)~~ from 6 months to twenty four months.

The other point of interest is that when columns (i) and (iii) in either table were compared, it was evident that germination was better



in *iiia* than in *ia*, whereas germination was markedly higher in *id* than in *iiid*. This shows that spores stored at  $0^{\circ}\text{C}$  kept better during the first six months, but longer storage at that temperature was not as favourable for the viability of the spores as when they were kept at room temperature.

Percentage Germination of Spores showing

Effect of Age and Storage Germination

A. After 8 hours Incubation.

| Months<br>Storage | (i)<br>Bottle<br>$20^{\circ}\text{C}$ | (ii)<br>Bag<br>$20^{\circ}\text{C}$ | (iii)<br>Bottle<br>$0^{\circ}\text{C}$ | (iv)<br>Bag<br>$0^{\circ}\text{C}$ |
|-------------------|---------------------------------------|-------------------------------------|----------------------------------------|------------------------------------|
| a. 6              | 85%                                   | 88%                                 | 88%                                    | 92%                                |
| b. 12             | 82"                                   | 84"                                 | 62"                                    | 87"                                |
| c. 18             | 62"                                   | 65"                                 | 42"                                    | 48"                                |
| d. 24             | 46"                                   | 50"                                 | 25"                                    | 29"                                |

B. After 16 hours Incubation.

| Months<br>Storage | (i)<br>Bottle<br>$20^{\circ}\text{C}$ | (ii)<br>Bag<br>$20^{\circ}\text{C}$ | (iii)<br>Bottle<br>$0^{\circ}\text{C}$ | (iv)<br>Bag<br>$0^{\circ}\text{C}$ |
|-------------------|---------------------------------------|-------------------------------------|----------------------------------------|------------------------------------|
| a. 6              | 89%                                   | 92%                                 | 91%                                    | 96%                                |
| b. 12             | 85"                                   | 83"                                 | 67"                                    | 91"                                |
| c. 18             | 62"                                   | 66"                                 | 47"                                    | 56"                                |
| d. 24             | 50"                                   | 54"                                 | 28"                                    | 32"                                |

A comparison of columns i and ii in both tables shows clearly that spores kept in powder form germinated less rapidly than those which were stored while still in the anthers. Similar deductions could be drawn

by comparing columns iii and iv in both tables.

#### Effect of Light on Chlamydo-spores Germination.

Fischer (1957) reported some workers as to have found a direct effect of light on the germination of some smut chlamydo-spores. In the experiments performed to show the effect of temperature on spore germination, the spores were incubated inside a refrigerator where they were virtually in complete darkness, yet germination was very profuse, and there was no appreciable difference in the rate of germination between spores inside or outside the refrigerator when the same temperatures were employed. However, two sets of experiments were arranged in the manner described earlier. In one set, the slides were placed in petri-dishes that were exposed to day light. The other set of slides were placed in similar petri-dishes and kept in darkness. The two sets of spores were allowed to germinate for 8 hours before they were taken out and fixed immediately.

The same procedure was repeated for 16 hours making sure all the time that the temperature was the same in all the cases. The results showed that there was not in fact any significant difference in the rate of germination between those spores kept in darkness and those kept in light. Similar results were obtained in other similar experiments.

Thus it may be concluded that for this smut, at least, light has no definite part to play in chlamydo-spore germinations.

#### Effect of pH of the Medium on Chlamydo-spore Germination.

Fischer (1957) reports that the acidity of the medium strongly influences the extent of germination in all species of the smut fungi, but the cardinal points varied from one species to another. The pH range for most species is generally very wide. Ling (1960) found that for Urocystis occulta, the optimum pH was 6.6, while germination at varying degrees was observed at pH.4 as minimum and pH.8.95 as maximum.



Although pH. values are available for only a few species of smuts, it is apparent from the literature that in most of the reported cases the optimum pH. for germination lies to a greater degree in the acid side. This is also true for most other fungi.

Experiments were performed to investigate the effect of pH. of the medium on the germination of chlamydospores of U. violacea.

The buffer solutions were prepared following the procedure recommended by Dawson (1959), using two phosphate compounds A and B. 0.2 molar solutions were prepared from each. One of the two hydrated forms was chosen from each compound.

Certain calculated volumes from each of the two liquids were mixed and then diluted up to 200 ml. with distilled water. The pH. for each mixture was then checked in a pH. meter. The following table gives the pH. values ranging from pH. 5.8 to pH. 8 which were used in these experiments. The two phosphate salts used were  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  and  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ .

Table Showing Different Volumes of the Phosphate Solutions.

| pH. | 0.2M<br>$\text{Na}_2\text{HPO}_4$<br>ML. | 0.2M<br>$\text{NaH}_2\text{PO}_4$<br>ML. | pH  | 0.2M<br>$\text{Na}_2\text{HPO}_4$ | 0.2M<br>$\text{NaH}_2\text{PO}_4$ |
|-----|------------------------------------------|------------------------------------------|-----|-----------------------------------|-----------------------------------|
| 5.8 | 8.0                                      | 92.0                                     | 7.0 | 61.0                              | 39.0                              |
| 6.0 | 12.3                                     | 87.7                                     | 7.2 | 72.0                              | 28.0                              |
| 6.2 | 18.5                                     | 81.5                                     | 7.4 | 81.0                              | 19.0                              |
| 6.4 | 26.5                                     | 73.5                                     | 7.6 | 87.0                              | 13.0                              |
| 6.6 | 37.5                                     | 62.5                                     | 7.8 | 91.5                              | 8.5                               |
| 6.8 | 49.0                                     | 51.0                                     | 8.0 | 94.7                              | 5.3                               |

(All solutions made up to 200ml. in distilled water).

Germination tests performed in the normal way at  $20^\circ\text{C}$ . The duration of each experiment was 10 hours. It was possible to set up spores for germination in solutions of three different pH. values at one time and four readings for each pH. were taken.

The results as deduced from the following table show that the pH. range taken could have been extended especially at the acid side, since even with the lowest pH. of 5.8, germination was 54% at that temperature and duration. The optimum pH. for germination as deduced from these data is pH. 6.6, the maximum was slightly more than 8 and the minimum in the neighbourhood of pH. 5.2 deduced by extrapolating a graph plotted to show this relation. ( Fig. I , Plate IV )

Thus the slightly acid conditions with a pH. of 6.6 are most favourable for the spore germination of U. violacea.

Spore Germination after 10 Hours in Different pHs.

| pH. | Total number of Chlamydospores obtained | Total number of Chlamydospores that had germinated | Percentage Germination |
|-----|-----------------------------------------|----------------------------------------------------|------------------------|
| 5.8 | 112                                     | 66                                                 | 54                     |
| 6.0 | 126                                     | 79                                                 | 63                     |
| 6.2 | 148                                     | 106                                                | 71                     |
| 6.4 | 115                                     | 92                                                 | 80                     |
| 6.6 | 113                                     | 96                                                 | 85                     |
| 6.8 | 108                                     | 89                                                 | 83                     |
| 7.0 | 116                                     | 93                                                 | 80                     |
| 7.2 | 122                                     | 90                                                 | 73                     |
| 7.4 | 112                                     | 78                                                 | 70                     |
| 7.6 | 126                                     | 85                                                 | 67                     |
| 7.8 | 111                                     | 45                                                 | 41                     |
| 8.0 | 101                                     | 22                                                 | 22                     |

Sporidial Fusions in U. violacea

Many of the earlier workers, notably Brefeld, De Bary, Plowright, Dangeard and Harper had noticed that when spores of U. violacea were grown in nutrient media, they germinated after a very short time. The promycelia budded off sporidia which in turn budded off secondary and tertiary sporidia. The process continued on for as long as there was



nutriment in the medium. Plowright (1889) reports that Brefeld, working on U. sagetum, managed to keep sporidia of this species, which is closely related to U. violacea, reproducing themselves for more than a year, by replacing the nutrient fluid as it became exhausted.

This was also found to be true in the case of U. violacea sporidia. When the chlamydospores were germinated in nutrient agar or any other nutrient medium employed in this research, it was found that sporidia were produced in great numbers and that budding was so profuse in many cases that the sporidia actually formed long chains and colonies of yeast-like cells.

Three different concentrations of malt extract 1%, 2% and 5%, a similar series of glucose, and distilled water were used as media for the germination of chlamydospores and subsequent observations on sporidial fusions. For each of these seven liquids, the chlamydospores were placed in sterilised glass wells together with about 1.c.c. of the liquid.

The systems were then incubated at two temperatures - 19°C and 25°C for a period of 96 hours. For each liquid and at each temperature four glass wells were used.

After incubation, a drop of the liquid was placed on a glass slide together with a drop of cotton blue and the preparation was examined for sporidial fusions. To compare the behaviour in the different liquids, and the effect of the temperature on the whole process, percentages were drawn out showing the number of sporidia in the field and the numbers that had formed conjugating pairs.

Table Showing Conjugation of Sporidia  
on Different Nutrient Media after  
96 Hours

| Medium          |      | A   | B  | Percentage<br>B/A |
|-----------------|------|-----|----|-------------------|
| Distilled water | 19°C | 100 | 34 | 34                |
|                 | 25°C | 85  | 32 | 39                |
| 1% Glucose      | 19°C | 94  | 19 | 20                |
|                 | 25°C | 116 | 27 | 23                |
| 1% Malt Extract | 19°C | 112 | 21 | 19                |
|                 | 25°C | 92  | 20 | 22                |
| 2% Glucose      | 19°C | 108 | 15 | 14                |
|                 | 25°C | 95  | 15 | 16                |
| 2% Malt Extract | 19°C | 79  | 10 | 13                |
|                 | 25°C | 94  | 13 | 14                |
| 5% Glucose      | 19°C | 115 | 14 | 14                |
|                 | 25°C | 87  | 13 | 15                |
| 5% Malt Extract | 19°C | 74  | 9  | 12                |
|                 | 25°C | 100 | 13 | 13                |

A = Total number of Sporidia.

B = Conjugating Sporidia.



At the higher temperature ( $25^{\circ}\text{C}$ ) a higher percentage of fusions was observed as compared to the lower one ( $19^{\circ}\text{C}$ ). The sporidia were generally slightly larger and more pointed at the higher temperature.

Conjugations were inversely proportional to the concentration of the media, because vegetative growth continued until the medium was exhausted and only then could conjugation occur. Thus a higher concentration of medium allowed a longer vegetative period, and therefore observations at some fixed time, e.g. 96 hours, would reveal fewer conjugations with higher nutrient content.

It was noticed that in all concentrations of glucose and malt extract, fusions were more frequent in glucose than in the extract and in both cases there was a gradual increase in the percentage of conjugations with decrease in concentrations of the nutrients and conjugations were greatest when distilled water was used as the medium.

The above facts could be explained in the following way:-

- (1) The higher temperature must have increased the kinetic energy of the sporidia, which were no doubt jostling about, and therefore increased the chances of two sporidia of different mating types coming together and conjugating.
- (2) Glucose seemed to be more readily assimilated and therefore took a shorter time to be used up with the result that in general, there were more conjugations at a given glucose concentration as compared to the equivalent concentration of malt extract.
- (3) Conjugation at 96 hours was inversely proportional to nutrient concentration, since the process itself is an end to vegetative growth which is terminated when the nutrient is depleted.

The nature of conjugations varied. In some instances, the two conjugating sporidia, each sent out a conjugation tube, the two tubes joined together and their walls coalesced thus establishing the continuity of the cell contents of the two sporidia. In other cases, the conju-



gation tube apparently came from one cell. The conjugation tubes originated at small depressions in the sides of the sporidia, which then looked like two kidneys joined together (Fig. III Plate VII). Conjugation tubes sometimes joined the two pointed ends of the sporidia or one pointed end of one to the side of the other (Fig. I Plate VII). In fact there was no definite pattern for these fusions, and conjugation tubes could emerge from any points on the walls of the sporidia.

Conjugations also occurred between sporidia and promycelial cells in the same way as with other sporidia.

A point of great cytological significance is that fusions as far as could be observed, took place between two cells only and never between more than two cells. This would mean that after fusion has taken place, a physiological barrier must have been created which prevented any more fusions taking place. This is comparable to the state of affairs in fertilization in higher forms of life and this is perhaps why De Bary referred to these fusions between sporidia as being sexual and hence the controversy between him and Brefeld reported earlier.

The cytological investigations which are described below confirmed that although the fusions could be regarded as a sexual behaviour between sporidia, there was no actual migration of nuclei from one cell to another. Therefore these conjugations would be described as a coming together of two cells of different mating types as a prelude to another step which in this case is deferred until the maturation of the chlamydospore.

#### Cytological Studies on Spores and Promycelia.

Dangeard (1892) studied the nuclear phenomena in a number of species of Ustilago and some other genera of the smut fungi. He found that the young spore had two nuclei which later united and fused into one nucleus. He considered the fusion of these two nuclei as equivalent to male and female nuclear conjugations. It is assumed that he was in agreement



with De Bary as regards the sexual nature of these fusions between sporidia. He also found that the sporidia were uninucleated.

Harper (1899) used some species of Ustilago as material for the study of the nuclear behaviour in smuts. His findings agreed with those of Dangeard.

Wang (1932) examined the fixed and stained chlamydospores which were at the point of germinating. She claims to have observed nuclear divisions and that the haploid chromosomes number for U. violacea was two.

A few fixatives and stains recommended for smut fungi were used for both chlamydospores and sporidia. Harper had used Flemming's triple stain preceded by a fixation in Flemming's weaker fixative of chromic, acetic and osmic acids.

Harper's method, with slight adaptations was tried and produced some good results.

Chlamydospores were first germinated in distilled water and kept there for four days until there was a high percentage of sporidia fusing in pairs. Slides were then cleaned in absolute alcohol, dried completely and covered with a very thin film of glycerine albumen. The slides were heated over a gentle flame to make the albumen adhesive. Very tiny drops of the liquid containing the germinating spores were deposited from a very fine pipette onto the slide. About nine such drops were deposited on an area that could be covered by a cover glass. A similar fine pipette was used for depositing the fixing fluid on the spores after the latter had lost most of the water around them. The drops of the fixative had to be as small as possible otherwise it was found that the spores were washed away in subsequent treatments. The spores were kept in the fixing fluid for 30 minutes; the fixative was then washed away and the slides passed through the normal series of alcohols and Flemming's triple stain and then mounted in euparal.

1. The obvious disadvantage of this staining method was that it was too long and although it did stain the nuclei in the promycelial cells and sporidia, the cell cytoplasm also took the stain through to a lesser degree, thus making observations on the nuclei somewhat difficult. The chlamydospores with their thick walls, were not stained at all. For these reasons this method was abandoned and other fixatives and stains were employed.

## 2. Propionic Orcein

The germinated spores were fixed in 3% aqueous alcohol, cleared in cedar oil for six hours, fixed onto the slides and hydrolysed in 10% KOH for 7 minutes at 60°C. Spores were washed in 4% propionic acid which was then washed away and replaced by propionic orcein. Slides were covered slightly and sealed.

When permanent preparations were required, the covers slides were floated off in 4% propionic acid and the material was dehydrated in two changes of each of 70% and absolute alcohol and mounted in cedar oil.

Propionic acid was found to be superior to acetic acid in clearing stains because of the clearer preparations obtained and the almost uniform background as compared to the granular background of acetic acid.

## 3. Azure "A"

The third stain - Azure "A" - used in these experiments was provided by Remond's fixative. This fixative developed by Remond (1935) was originally used for chick embryos, *Chlamydomonas* and *Algae* but it was found to be a very stable mixture that could be stored for long periods.



### 1. Heidenhain's Haematoxylin

This stain was preceded by fixation in Bouin's fixative in some preparations and by Helly's fixative in others.

The preliminary preparations of the slides were the same as for previous experiments. The spores were germinated in distilled water in glass wells and fixed before they were transferred to the albuminated slides. Small drops of the fixative containing the spores, when deposited on the slides, were found to cause some solification<sup>d</sup> of the albumen which was further hardened by gentle heating. Mordanting in iron alum and staining in haematoxylin were each allowed for 24 hours. The material which at first looked all black, was destained in iron alum and watched under the microscope. Dehydration was followed by mounting in Euparal.

### 2. Propionic Orcein

The germinated spores were fixed in 3:1 absolute alcohol glacial acetic acid for six hours, fixed onto the slides and hydrolysed in N/10 HCl for 7 minutes at 60°C. Spores were washed in 45% propionic acid which was then washed away and replaced by propionic orcein. Slides were warmed slightly and sealed.

When permanent preparations were required, the cover glasses were floated off in 45% propionic acid and the material was dehydrated in two changes of each of 95% and absolute alcohol and mounted in Euparal.

Propionic acid was found to be superior to acetic acid in the orcein stain because of the clearer preparations obtained and the almost colourless background as compared to the granular background in Aceto-orcein.

### 3. Azure "A"

The third stain - Azure "A" - used in these experiments was preceded by Newcomer's fixative. This fixative developed by Newcomer (1953) was originally used for chick embryos, Tradescantia and Allium root tips. It was found to be a very stable mixture that could be stored for many months.



The slides were prepared in the usual way. Spores were deposited on the albumen. The liquid around them was allowed to evaporate but not to dryness. A very small amount of the fixing fluid was dropped on the spores. This was enough to cause the albumen to coagulate sufficiently to fix the spores in position. The slides were then transferred into jars containing the fixing fluid where they were left for 10 minutes.

Hydrolysis in N.HCl for 7 minutes at 60°C was followed by staining in Huebschman's Azure "A" for a minimum period of 4 hours; it was found that longer periods in the stain produced even better results especially with the chlamydospores.

<sup>6</sup>  
Huebschman's Azure A which was developed by Robinow (1957A and 1957B) and used with various yeasts, was found to be very suitable in staining spores of U. violacea especially the sporidia.

Thorough dehydration followed by prolonged clearing in clove oil -- at least 30 minutes -- gave the best results.

An attempt was made to find some spores on the point of germination, when it is reputed that, for most smut fungi, nuclear division takes place. Some spores were germinated in distilled water and the process was checked by fixation after every half hour from 4 to 8 hours. At each time some of the spores were stained in Azure A and observed. A wide range of spores at various stages of development was thus prepared.

The nuclear behaviour in very young spores was also examined. Such very young spores were difficult to obtain, because spores in their earlier stages of formation were required. After some trials, it was found that such young spores were enclosed in the mucilaginous matrix which covered the whole mycelium before the latter was broken up into small sections which later on formed the chlamydospores. Very young anthers -- ones that had just started to develop the normal pink colouration -- were taken from infected buds. Using very fine forceps and dissecting needles, an anther was split open and the pinkish gelatinous mass were cut into very small



pieces. These little pieces of material from the anthers were placed on slides smeared with glycerine albumen and flattened down by squashing under cover slips.

Subsequent treatments with regard to fixation and staining in Azure "A" were the same as for chlamydospores mentioned above. The only difference here was that hydrolysis had to be extended to 9 minutes instead of the usual 7 minutes -- this was perhaps due to the presence of the mucilaginous sheath around young spores.

In some of the spores which were fixed at the point of germination, the nuclei appeared to be elongated as distinct from nuclei observed in other preparations where they were more or less rounded. Nuclear division in such spores was observed (Figure 1 Plate XVI). The shape and outline of the nucleus suggested that the chromatin was divided into four groups. Closer study of these dividing nuclei under phase contrast revealed that the four groups of chromatin material were actually chromosomes (Figure 1 Plate XVI). These four chromosomes were also observed when the nucleus in the first promycelial cell divided to furnish the second cell with a nucleus.

It became evident that the nucleus in the chlamydospore divided to give the nuclei in the promycelial cells.

Wang (1932) claimed that in *H. violaceum*, the haploid number of chromosomes was 2. This is further confirmed by our findings as mentioned. Therefore the chromosome complement of *H. violaceum* is 2.

In preparations where there was only one promycelial cell, it was observed that the nucleus was at the tip of the cell. Later on it was seen occupying a more central position in the cell. This was the formation of the second cell. The new nucleus was visible when the third cell was formed.

## Results

Microscopic examinations, of the prepared slides, under oil immersion revealed the following facts:

1. In the mature chlamydospore there was one large central vacuole enclosed in a layer of cytoplasm varying in its thickness. (Figure 11 Plate XIII ). Embedded in the cytoplasm, there was a clearly defined nucleus occupying different positions in the various spores examined. These resting nuclei varied in size from 1 to  $1.5\mu$ . A single darkly staining nucleolus was very distinct in some of the preparations.

In some of the spores which were fixed at the point of germination, the nuclei appeared to be elongated as distinct from nuclei observed in other preparations where they were more or less rounded. Nuclear division in such spores was observed (Figure 1 Plate XVI ). The shape and outline of the nucleus suggested that the chromatin was divided into four groups. Closer study of these dividing nuclei under phase contrast revealed that the four groups of chromatin material were actually chromosomes (Figure 1 Plate XVI ). These four chromosomes were also observed when the nucleus in the first promycelial cell divided to furnish the second cell with a nucleus.

It became evident that the nucleus in the chlamydospore divided to give the nuclei in the promycelial cells.

Wang (1932) claimed that in U. violacea, the haploid number of chromosomes was 2. This is further confirmed by the findings just mentioned. Therefore the chromosome complement of U. violacea is 4.

In preparations where there was only one promycelial cell, it was observed that the nucleus was at the tip of the cell. Later on it was seen occupying a more central position just before division and the formation of the second cell. The same process was repeated when the third cell was formed.



It appears, therefore, that the first nuclear division took place within the spore. One of the two nuclei resulting from this division migrated into the germ tube where it divided once to give the two nuclei in the promycelium. The nucleus in the distal cell also divided once to give two nuclei -- one migrating into the third promycelial cell.

2. Young chlamydospores had much thinner walls formed of only the entin. The vacuole, where visible, was much smaller than in the mature spore. Each young spore had two very clearly defined nuclei occupying a near central position and about  $1\ \mu$  apart. As these spores matured, their cell walls became thicker and the nuclei were drawn closer together (Figure 1 Plate XIII ). The preparations showed different stages in the maturation of the spores. Nuclear union -- fertilization -- occurred always before the laying down of the thick outer wall. This was clearly observed in some young spores which still had thin hyaline walls, but possessed single nuclei. The inverse situation i.e. two nuclei in a thick walled cell was never seen. Thus it was confirmed, here, that the young spores have two nuclei which fuse to form single nuclei in the mature spores.

3. When the spores germinated, promycelia with three cells were formed. There was a single nucleus in each promycelial cell. Examinations of stained sporidia also revealed that these too had single nuclei (Figure 1, II Plate XV ). It was observed that sporidia, apart from fusing with one another in pairs, sometimes fused with promycelial cells. This clearly indicated that the sporidia and the promycelial cells must necessarily have the same nuclear types i.e. both must be haploid.

4. When spores remained in the germinating fluids for more than 4 days before being fixed and stained, it was found that many of the sporidia had joined up in pairs. This fusion however, was only somatic, since in all the cases examined the nuclei in the pairing sporidia remained

in their respective cells. It was observed that the nuclei migrated towards the bases of the conjugation tubes but they never actually moved into them; this step evidently takes place inside the host tissues where the dikaryotic mycelium is established.

From the foregoing experiments and observations the nuclear phenomena in U. violacea could be summarised as follows:-

The mature chlamydospores, which are formed only in the anthers of infected flowers, are uninucleated and represent the only diploid stage in the whole life history of the fungus. When these spores germinate, they produce promycelia whose cells are uninucleated and haploid. The promycelia bud off sporidia which are also uninucleated and haploid and which fuse in pairs through conjugation tubes. The nuclei of the two members of a pair associated but remain separate even after the formation of the chlamydospores, which start as being binucleated. As these spores mature, their nuclei fuse together to form the single diploid nucleus seen in the mature chlamydospore.

Anatomical investigations of these parts of the plant were the specialism were expected to be detectable type material. Different combinations of fixatives and stains were used to determine the extent of the mycelium in the host tissue.

Various parts of the infected plant were fixed in different fixatives. The duration in each fixative depended largely on the nature of the fixative and on the size of the plant material used. With very young buds, the time in the fixative was shortest, while it was longest for parts of the plant which were older.

After being fixed for varying periods in the different fixatives, the plant parts were stored in Jeffrey's fluid made up of equal parts of glycerine, absolute alcohol and distilled water. The change to this fluid, apart from making it possible to perform experiments at any convenient time, helped to soften the tissues.

The pieces of plant material were then dehydrated in the usual alcohol series and then through mixtures of alcohol and chloroform.



### The Parasitic Mycelium.

Sampson (1939) reports, that in all smuts, the parasitic mycelium is dikaryotic. This view was deduced from the fact that where sporidial fusion was known to occur, monosporidial cultures failed to cause infection.

In the literature, the mycelium of U. violacea is reported to be systemic which implies that hyphae should be detected in all parts of the host plant.

The infection experiments, which were reported earlier in this work, show that there was no definite point of entry through which the fungus establishes itself in its host plant i.e. infection could occur through flower, bud or even by spores' germ-tubes attacking the younger parts of the growing shoot. The ultimate result of such infections was always the production of chlamydospores in the anthers. One would therefore expect to find the mycelium in the tissues lying between the point where infection took place and where the chlamydospores are formed.

Anatomical investigations of those parts of the plant where the mycelium were expected to be detectable were carried out. A number of different combinations of fixatives and stains were employed in revealing the extent of the mycelium in the host tissues.

Various parts of the infected plant were cut into small pieces and fixed in various fixatives. The duration in each fixation depended largely on the nature of the fixative and on the size of the pieces of plant material used. With very young buds, the time in the fixative was shortest, while it was longest for parts of the stem and root stocks.

After being fixed for varying periods of time in the different fixatives, the plant parts were stored in Calberla's fluid made up of equal parts of glycerine, absolute alcohol and distilled water. The storage in this fluid, apart from making it possible to perform experiments at any convenient time, helped to soften the tissues.

The pieces of plant material were then dehydrated in the usual alcohol series and then through mixtures of alcohol and chloroform.



The specimens were embedded in wax, and a few wax blocks were prepared in readiness for making microtome sections. Some of these microtome sections were 5 $\mu$  in thickness, others were 10 $\mu$ . Sections were then fixed to slides smeared with either glycerine albumen or Haupt's adhesive.

When required for staining, the wax was removed by leaving the slides in three changes of xylol and for two hours in each change. The slides were by then ready for any of the three staining procedures listed below, which were found most suited for the purpose of these experiments - detecting the fungus mycelium in the host tissue.

- i Safranin and Light green in Clove oil.
- ii Magdala red and Fast green F.C.F. in Clove oil.
- iii Heidenhain's haematoxylin.

A. Safranin and Light green in Clove oil.

The above stain although it was not designed for critical study of the mycelium in host tissue was tried here because of the quick results expected from it and which would be used as guiding lines to more specific stains.

Safranin was used in the form of 1% in 70% alcohol and the light green was 0.5% in clove oil.

The hyphae and the cellulose walls of the host were all stained green in contrast to the nuclei and xylem elements which were stained red. Differentiation was not adequate enough since the hyphae and the cellulose walls showed the same colour, but even then, it was possible to distinguish individual hyphae.

B. Magdala red and Fast green F.C.F. in Clove oil.

Johansen (1940) seems sceptical with regard to the use of Magdala red as a stain for plant tissues. He suggests that schedules calling for this dye give very different results with various lots of dye, and recommends its substitution with phloxin. In our case, this stain proved to be the best of the stains used when preceded with fixation in



Helly's fixative.

Various strengths of the dye were tried, and the one that gave the best results was a 2% solution in 80% alcohol. Since the solubility of the dye was greater in water than in alcohol, two grammes of the dye was first dissolved in 20 c.c. distilled water which was then made up to 100 c.c. by the addition of absolute alcohol. 0.5% Fast green F.C.F. was prepared according to Johansen (1940).

Before passing the sections through the stain, it was found that to a very great extent the quality of the preparations depended on whether or not there were any traces of wax on the slides. (Traces of wax resulted in red patches in the preparations).

Sections were stained in solution "A" - Magdala red for 10 minutes and in the counter stain for only 5 minutes. It was found best to wash the sections in clove oil after staining in fast green and give them only a very brief rinse in absolute alcohol before mounting in Euparal otherwise the sections all looked pink.

The results showed that the host tissues with the exception of the nuclei and in some cases the chloroplasts were stained bluish-green whereas the hyphae especially where they were gelatinised - at or near the anthers - were stained very bright red.

#### C. Heidenhain's Haematoxylin.

This stain, recommended and used by Wang (1932), produced fairly good results especially with sections of buds or very young parts of the shoot.

Sections were first fixed in Bouin's fixative prepared as in Gurr (1963). After the removal of wax, sections were mordanted in iron alum in the usual way and stained in Heidenhain's haematoxylin prepared and bottled in Messrs. G. T. Gurr laboratories. Clearing was done in clove oil and mounting in Euparal. No counter stain was necessary.

The hyphae were more sensitive to the stain than the host tissues and they remained darkly stained even after the host cells were almost completely destained.

Extent of the Mycelium as Observed with the Three Staining Methods  
as Described Above.

(i) Sections through Anthers.

In very young anthers, there were masses of undifferentiated parenchyma occupying the centres of the anther lobes. At the corners, there were traces of mycelium which stained bright red when stained with Magdala red and dark brown when stained with haematoxylin. In slightly older anthers, these mycelial threads were thicker, gelatinous looking and they occupied more space at the expense of the anther tissue. (Fig. 1 Plate XIV). It was observed that these mycelial threads grow round the cells filling up the intercellular spaces at the same time growing towards the centre of the anther lobe. It was observed that although the hyphae first appeared close to the anther walls, the youngest hyphae were also observed there. This is because as the mycelium aged, it spread to the centre of the chamber and continued its growth and maturation from that point, then spreading outwards. The result was that in very old anthers, it was observed that the centre of the anther chamber was formed entirely of smut spores, whereas just inside the anther walls, there was an empty space.

In old anthers the hyphae were split into very small units enclosed in a mucilaginous sheath which disappeared when these units developed into (mature) chlamydospores.

The infected anthers as seen in cross section or longitudinal section, had developed the same layers including the tapetum which, however, was destroyed by the invading fungus. (Fig. 11 Plate XI).



(ii) Sections Through other Parts of Infected Buds.

The hyphae as seen in parts of the buds other than the anthers, were very thin and ran parallel to the conducting tissues. At the base of the ovary, the hyphae were very abundant in the intercellular spaces, and pushed the cells aside thus distorting their general layout. Although haustoria as such were not detected, invaginations in the cell walls adjacent to the hyphae were clearly seen (Fig. [Plate XII]). Where the hyphae were very sparse, there was no interference with the normal growth of the host cells. The hyphae were so thin that any details as regards presence or absence of clamp connections could not be observed, although these had been reported in some species of smuts, but not in U. violacea.

iii) Sections Through Pedicels and Younger Parts of Stem.

The hyphae in these regions were difficult to find, since they were very thin and ran parallel and close to the vascular system. In many of the cases observed, they were present in the intercellular spaces of the xylem and phloem parenchyma, but they were also found inside the xylem vessels. Individual hyphae could not be followed for more than 25 $\mu$  without changing the focussing of the microscope. This was due to the irregular shape of the hyphae and their meandering path. The thickest sections of 10 $\mu$  would not show more than 60 $\mu$  of the hyphal length since it was not all at the same level. The host cells adjacent to the hyphae seemed, in longitudinal sections, to have been slightly affected by the fungus, since they were more sensitive to the stain especially haematoxylin, than other cells further away from the hyphae.

In the root system, all attempts to reveal the mycelium failed. Baar (1903) working on Silene pratense reported that he had found the "permanent" mycelium in the root stocks. He employed Magdala red as the stain but did not specify the fixative used.

It appears, from the above, that at least all parts of the axis in the shoot system were possible locations for the detection of the mycelium, though hyphae were much more frequently found at points close to the anthers where the resting spores are normally formed. Away from the flowers, the mycelium was more abundant in the nodes than in the inter-nodes.

In healthy plants of *Silene dioica*, the distinction between a male and a female flower is an easy matter and does not need stopping to dissect and look into the internal organs.

Both flowers are actinomorphic. The calyx is gamosepalous below a glandular hairy tube. The calyx tube widens upwards and narrows towards the top into a toothed structure. The main difference here between the staminate and the pistillate flowers is in the extent to which the calyx tube widens in the middle; in the staminate, it is slender and cylindrical while in the pistillate flower, the calyx tube widens to such an extent that its width becomes almost equal to its length.

In the corolla there is no real difference between the two flowers except in the size of the horizontal part of the corolla tube which the calyx sits.

In the female flower there is one ovary and there are stamens and rudimentary stamens. In these rudimentary stamens the filaments are well developed and partly developed while the anthers are small, but the anthers in the male flowers, nevertheless, in their surrounding tissue are not for anything else. In the male flower, there are ten stamens arranged in two whorls of five each. The outer whorl is opposite the sepals and the inner whorl is opposite the petals.

There are no special nectaries, but in the female flower, nectar is secreted from the base of the ovary and in the male flower, it is secreted from the base of the rudimentary ovary.

This, then, is a short description of the two flowers in their normal healthy states.



Infected FlowersOBSERVATIONS ON INFECTED FLOWERSNormal flowers

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In the corolla there is no real difference between the two flowers except in the size of the horizontal part of the corolla lying outside the calyx tube.

In the female flower there is one ovary and there are always ten rudimentary staminodes. In these staminodes the filaments are very short and poorly developed while the anthers though much smaller than anthers in the male flowers, nevertheless, in their morphology cannot be mistaken for anything else. In the male flower, there are ten stamens arranged in two whorls of five each. The outer whorl is opposite the sepals and the inner whorl is opposite the petals.

There are no special nectaries, but in the female flower, nectar is secreted from the base of the ovary and in the male flower, it is secreted from the base of the rudimentary ovary.

This, then, is a short description of the two flowers in their normal healthy states.



### Infected Flowers

As a result of natural infection by Ustilago violacea, some of the differences between pistillate and staminate flowers are reduced to a very great extent. In the female, some of the characters <sup>which</sup> are related to maleness are exhibited and the reverse is also true, with the result that both flowers looked more hermaphroditic and more like one another. There are also some changes in the general appearance which are common to all infected flowers.

Before they open, the buds look distorted in shape as if they were buds of zygomorphic flowers. This is because of the unequal development of the internal organs - the stamens, whether natural as in the male flower or induced as in the female flower, are hypertrophied while the ovary is aborted in the female flower and further development is induced in the male flower. An infected flower is also detectable in the bud stage because of the split calyx referred <sup>to</sup> by many authors mentioned previously and observed as a constant feature. The explanation of this split calyx is that the petals elongate abnormally inside the calyx tube i.e. inside a limited space and therefore curving, and causing a bulge at one side. The strain at this bulging side causes it to split.

When infected flower buds were dissected, it was observed that all ten stamens were of the same length, whereas in the healthy male and especially at the bud stage, the stamens were clearly in two whorls and the filaments were longer in the inner whorl than in the outer one. The anthers were also at two different levels. In buds from healthy male flowers, the anthers were generally larger and more mature in the outer whorl than in the inner whorl and they dehisced earlier. But the anthers from infected flowers, whether male or female, all dehisced at the same time, three to four days before the flowers opened.

Another characteristic of infected flowers of both sexes is that the petals are pale in colour compared to the bright pink of the healthy flowers.



The above description applies to both naturally and artificially infected flowers. But when we consider plants that were artificially infected and compare them to the naturally infected ones, we find that there are some minor differences in the nature and extent of the infection. Firstly, naturally infected plants produce infected flowers in all their branches though in some of the branches, some healthy flowers may be produced especially towards the end of the flowering season. In artificially infected plants not all branches produced infected flowers. Secondly, in artificially infected plants (especially when mature plants were infected) sometimes not all the anthers in a male plant produced smut spores. Pollen grains were also found mixed with spores taken from infected male flowers. This had not been the case in the naturally infected flowers where all the anthers were full of smut spores and no pollen grains were found. These two differences could be caused by the difference in the duration of infection. In the naturally infected plants, infection tends to be systemic, whereas, in artificially infected plants, whole branches were observed bearing healthy flowers of either sex. When seedlings were inoculated, the resulting infected plants had smutted flowers in all their branches in the same way as in naturally infected plants. This indicates that natural infection in most cases must be through seedlings.

The table given in page 41 gives the results obtained from inoculating seedlings. Infected plants were quoted as such, without reference to their sexes. It was perhaps not necessary to state the sexes at the time, since we were only interested in the percentage of plants infected. But as was mentioned previously, an infected male flower has certain characteristics to distinguish it from an infected female flower. To be absolutely sure about the sex genotype of an infected plant, root-tip squashes must be made.

The roots from infected plants were obtained in one of two ways:-



(1) Cuttings were made at the bases of the plants and placed on plastic trays with holes at the bottom. The lower parts of the cuttings were allowed through the holes. The plastic trays were then placed on a large tank full of distilled water. The lower parts of the cuttings were dipping in the water. An air pump was employed to give the submerged parts of the cuttings a continuous supply of air. These experiments were repeated three times and each time there were three trays on the tank and on each tray there were eight cuttings from eight different infected plants. This gave a total of seventy two plants' cuttings. (Plate XIX )

After four to five weeks in each case, there was a rich crop of actively growing roots which were then taken out and treated by the two staining squash methods - the Feulgen method and cresyl violet stain. The following results were obtained:-

Of the seventy two plants examined, forty three were male and twenty nine were female.

(2) The other technique employed in obtaining young healthy roots for squashes from potted plants was as follows:-

The potted plant was flooded with water in the morning. In the afternoon, when most of the excess water was drained off, the pot was inverted and with only a gentle tap, the whole soil slipped off. Very healthy looking roots - white and translucent were picked out from the surface of the soil. These were obviously the roots getting the greatest amount of aeration and, therefore, judging from their healthy growth and appearance, containing many dividing cells. The plants used in (2) were twenty four of the seventy two plants used in (1). The results in (2) were a confirmation of the results in (1) with regard to the twenty four plants used.

The results obtained in these experiments were further confirmation that female plants of S. dioica are less susceptible to attacks of



U. violacea than male plants.

A group of experimentally infected plants now growing near the laboratories in this Department, have among them a healthy female plant and for two successive seasons this plant has produced only healthy flowers. A male plant under the same conditions produced healthy flowers in the first season (1964) only, but this summer most of its branches were bearing infected flowers.

#### Stability of Sex in *Silene dioica*

In October 1962, two healthy male plants and two healthy female plants were transplanted from Stravithie and kept in the greenhouse at 65-70°C. These four plants have kept producing flowers all year for the last three years. The two male plants produced only male flowers and the female plants produced only female flowers.

About fifty plants raised from seed and kept in the greenhouse under the same conditions, produced either male or female flowers according to their sex. There was no incidence of hermaphroditism.

Two plants also raised from seed behaved differently. In these two plants, every first blossom in a flowering shoot was female while all the others were male. This phenomenon was almost overlooked at the beginning, because although these female flowers did eventually produce capsules from which viable seeds were obtained, the shape of the flower was very much like that of a male flower. Even the capsules from which seeds were collected, did not look like ordinary capsules - they were long, cylindrical and shaped like calyx tubes in normal healthy male flowers. The seeds were less than half the size of seeds obtained from normal female flowers.

All the available seeds were collected from these capsules and plants were raised from them.

The total number of seeds collected from all the eight capsules was 152. This number is much smaller than that expected from eight capsules (c.f. root-tip squash experiments). Of these only 88 or under 60%, were viable, and of these only 49 of the seedlings survived. These points are perhaps of some interest, since in other experiments using seeds from normal females, there was a 100% germination in every case.

Flowering has not yet taken place in all the plants raised - which is unusual, because normally 12 months is ample time for flowering to take place and these plants have been growing for 16 months now. However, of the few plants that had flowered, there was no incidence of that leading female flower. (All the six plants that have so far flowered were male). Cytological examinations of root tips using both the Feulgen and propionic orcein methods revealed that in both plants - with the leading female flowers - the chromosome complements were the same as those typical for male plants; there were 24 chromosomes with two differently shaped sex-chromosomes ( $2A + X + Y$ ). This test of course is true of the root tip cells or the apical meristem. But other cells such as those of the embryo sac and pollen mother cells may behave differently under abnormal conditions. In this case the environmental conditions may have influenced the pollen mother cells by suppressing their activity and encouraging the development of the rudimentary ovary and embryo sac mother cells in it.

Warmke (1946) discovered some plants which were normally male, but which produced occasional hermaphrodite flowers. But his plants, obtained through calculated crossings, had the chromosome complements of  $2A.XXY$ , and this is not exactly the same state of affairs in our case where the chromosome complement is normal.

The phenomenon was not observed in the large selection of field plants examined previously for the establishment of the sex ratio in S. dioica. But in the greenhouse where the total number of healthy plants was just over 50, there were two plants exhibiting this abnor-



### MANIPULATION EXPERIMENTS

malinity. One would have thought that since the chromosome complement was normal, the behaviour of the plants could be attributed to the influence of the environment. These plants were kept in the greenhouse where the temperature ranged from 65 to 70°F and artificial illumination was maintained for 14 hours a day. If this explanation is validated, then it would support Hillman's view (1964) on the effect of light on flowering and its bearing on sex expression.

Experiments were performed where all the anthers were removed from healthy male flowers and ovaries from healthy female flowers. The procedure was as follows:-

A potted plant was placed on a revolving platform about 100 cm. in diameter. Flowering branches were pulled aside and pinned down in turn. About 10 young buds - ones that would normally have opened after 6 or 7 days, were selected from each plant.

Employing a dissecting microscope for observation, a pair of very fine forceps and dissecting needles were used to make a small slit on one side of the bud. The slit was in the lower part of the bud, measuring  $1 \times \frac{1}{2}$  mm. The sides of the slit were made as smooth as possible so that the cut part could be flapped back to and with the bud when it opened.

The petals were then pushed aside and the stamens were removed. The petals were then returned to position and the bud was sealed with a very thin film of vaseline. The same procedure was applied to the case of female flowers where ovaries were removed.

The above emasculation experiments were continued over a period of three months.

Results of these experiments proved that anthers and ovaries were taken against desiccation and injury to the buds. The buds were kept in a moist atmosphere where the temperature was around 65°F.

### EMASCULATION EXPERIMENTS

Examinations of infected flowers revealed that in the infected female flowers, the stamens which in healthy flowers are rudimentary were well developed and the anthers hypertrophied. At the same time the ovaries aborted to the extent that the ovules never developed into seeds. In the infected male flower, apart from the fact that the pollen sacs were filled with smut spores instead of pollen grains stamen development seemed normal. Ovaries, though not fully developed were formed.

Experiments were performed where all the anthers were removed from healthy male flowers and ovaries from healthy female flowers. The procedure was as follows:-

A potted plant was placed on a revolving platform where flowering branches were pulled aside and pinned down in turn. About 12 young buds - ones that would normally have opened after 6 to 7 days, were selected from each plant.

Employing a dissecting microscope for observation, a pair of very fine forceps and dissecting needles were used in making a small slit on one side of the bud. The slit was in the form of a rectangle measuring  $1 \times \frac{1}{2}$  m.m. The sides of the slit were made in such a way that the cut part could be flapped back to one side exposing the organs underneath.

The petals were then pushed aside and the 10 anthers were picked out. The petals were then restored in position and the slit covered with a very thin film of vaseline. The same process was applied in the case of female flowers where ovaries were removed.

The above emasculation experiments were repeated twice over a period of three months.

Results of these experiments proved that despite all the precautions taken against desiccation and injury to the buds, the latter could not stand the shock. In most cases when the internal organs were removed,



### Photography, Microscopy and Radiography

the buds simply shrivelled off. In the few cases where flowers developed from these treated buds, there appeared to be no signs of what would have been the after effects of the removal of one set of the sex organs (anthers or ovaries) on the development of the opposite organs.

The sex of a flower is fixed long before the essential organs reach a stage of development at which they are recognisable and susceptible to manipulation in experimental techniques of the kind just described.

Photographs were taken with a Watson eye-piece camera on 35 mm. roll film, using a compensating x 10 eye piece and an apochromatic oil immersion objective. Light intensity was estimated by an electronic Watson light meter. The film used was Ilford 35-F roll - speed 50 and developed with D-11 developer. Prints were made on Kodak Bromide photographic paper.

Photographs of larger objects e.g. flowers or fruits were taken with an Ikon miniature camera fitted with range for close-up pictures.

### Photography, Microscopy and Photomicroscopy

Preparations were examined with the aid of a Watson Bactil Binocular Research microscope. For the stained preparations, the microscope was fitted with a 2 m.m. apochromatic oil immersion objective (N.A. 1.37, x 84), a universal No. 1 sub-stage condenser 10 m.m. (N.A. 1.0) and a x 10 compensated eye-piece. Blue or green sub-stage filters were used. The light source was a horizontal ribbon lamp (6v. 108 w.) controlled by a resistor and transformer from 240 v. A.C.

Microphotographs were taken with a Watson eye-piece camera for 35 m.m. roll film, using a compensating x 10 eye piece and an apochromatic oil immersion objective. Light intensity was estimated by an electronic Watson light meter. The film used was Ilford Pan-F roll - speed 50 and developed with ID-11 developer. Prints were made in Kodak Bromide photographic paper.

Photographs of larger objects e.g. flowers or graphs were taken with an Edixa miniature camera fitted with rings for close up pictures. The possible examination of all the parts of the large phyllodes for this work. But the fact that this found several directions members of the Caryophyllaceae and the in separate reflections on the sex expression of infected flowers, has posed an interesting challenge to workers on the phenomena of sex in these plants.

Like (1964), as mentioned previously, though that period of the large number of host plants that are mentioned in the paper, the species was not in fact a single species but a complex one which had to be divided into as many species as there were host plants. Our findings, and references to work by Fisher (1963) followed (page 6).

It is therefore possible that the system in the Caryophyllaceae proposed by Fisher and Shaw (1963) derives well towards the lower end of species to a smaller and suitable total. They suggested that a



two or more smut fungi of similar morphology and parasitizing the same or different species or genera of the same family and producing the same symptoms should be considered as belonging to the same species. Similarly smut fungi of similar morphology and producing similar effects on host plants belonging to different families should be treated as distinct species.

This system, if adopted, fits U. violacea well, since all the host plants belong to the same family. The symptoms are similar in all

### DISCUSSIONS

An examination of the literature reveals a very large number of species which act as host plants to the smut Ustilago violacea. The species listed on page 5 are only 35 out of the hundreds of species in the Caryophyllaceae, but these are the species where the smut had definitely been reported. Perhaps one reason why no larger section of the family is reported as being susceptible to the disease is that most of the plants in this family have no real economic importance, thus there is no incentive for studies of the matter from a wider angle e.g. the possible examination of all the genera of the Caryophyllaceae for this smut. But the fact that this fungus attacked dioecious members of the Caryophyllaceae and the subsequent influence on the sex expression of infected flowers, has posed an interesting challenge to workers on the phenomenon of sex in higher plants.

Liro (1924), as mentioned previously, thought that judging from the large number of host plants that are susceptible to the fungus, the species was not in fact a single species but a composite one and thus had to be divided into as many species as there were host plants. His own findings, and references to work by Zillig (1921) defeated his aim (page 6).

It is therefore possible that the system in the taxonomy of Smuts proposed by Fisher and Shaw (1953) serves well to reduce the large number of species to a smaller and workable total. They proposed that any

two or more smut fungi of similar morphology and parasitizing the same or different species or genera of the same family and producing the same symptoms should be considered as belonging to the same species. Similarly two smut fungi of similar morphology and producing similar effects on host plants belonging to different families should be treated as distinct species.

This system, if adopted, fits U. violacea well, when all the host plants belong to the same family, and the symptoms are similar in all hosts. [The last plant quoted in the list of host plants on page 5, does not belong to the Caryophyllaceae. If the observation of the smut were repeated and confirmed, then according to the proposed system above, this smut will have to be known under a different name].

Linnaeus (1737), as mentioned earlier, saw the infected flowers of Silene alba, but did not recognise them as such. Later workers, notably Giard (1888), tried to theorize the whole aspect of parasitic castration - or the influence of parasites (fungi) on the sexes of their host plants. But Giard considered that the influence of U. violacea on Silene dioica and Silene alba, is to induce some male characters in the otherwise female flowers. This is of course only half the truth, since it is not only the female flowers that are attacked by the fungus but also the male flowers.

Most of the earlier observers, some of whose work is reviewed by Fischer (1957), seemed to focus attention on the importance of attacks on female plants so that one felt that perhaps for some reason there was little attention on male plants. One reason which explains this is that the hypertrophy of the anthers in normally female plants is more pronounced than the development of ovaries in infected male flowers. The question is, therefore, about the relative importance of the hypertrophy of anthers in female flowers, and the development of abortive ovaries in male flowers.



It seems, from our own findings, that the general tendency for this fungus is to attack adult male plants of S. dioica; only in very rare cases does it attack adult female plants.

Seed transmission of the disease as a hypothesis advocated by Brefeld does not seem to stand the test, since all attempts to raise infected plants from smut treated seeds failed. The seeds were deliberately given a very heavy dose of spores and were germinated in conditions which were thought to have been most congenial to spore germination. But in an infected female plant, the ovary is castrated at an early stage in the development of the bud; therefore no seeds either healthy or infected are developed from the ovules. For the seeds to be the vehicles through which the fungus is transmitted, they must somehow be contaminated with the spores and such a situation cannot exist unless of course spores from outside were deposited on the capsules. Such spores should necessarily come from an infected plant. Thus from both theoretical and practical points of view, seed transmission of this disease is virtually impossible.

Insect transmission of the disease is the other method discussed in detail by various writers e.g. MacMillan (1892), Plowright (1892), Erickson (1930) and Baker (1947). The confusion in the statements of these authors is very evident. Apparently no distinction was drawn between transport of spores and the actual transmission of the disease. Whenever one reads about insects visiting infected flowers in search for pollen or nectar and flying away with their bodies covered with smut spores, one would immediately get the impression that these spores are later on deposited on healthy flowers and so infection is effected. Such a line of thinking would lead to the conclusion that the disease is essentially transmitted through the flowers - other modes of infection might, therefore, be of secondary importance. In the field, in heavily infected areas one notices some healthy plants growing in the midst of diseased plants and this continues for at least a whole season. If,



therefore, flower infection is so simple, then such plants would not be expected to produce healthy flowers towards the end of the season i.e. after many visits by potential spore carrying insects. At Kinkell braes, one plant observed there was infected while all the plants around it were healthy. This same plant produced diseased flowers in the next season and all the other plants remained healthy. This isolated case can be explained by the assumption that spores were carried to it by insects, since there was no other infected plant in the vicinity near enough to allow air born spores as another possibility. Flower infection cannot be ruled out since the author did find in artificial inoculations, that it is perhaps one of the certain ways of causing infection. But in nature conditions are not exactly the same as in the greenhouse in which spores are given optimum conditions for germination and the amount of the inoculum is ~~by~~ far greater than what one would expect in nature.

Some of the advocates of flower infection, e.g. MacMillan, believed that the spores germinated in the stigmatic juices and then invaded the rest of the flower. This hypothesis, if it were true, would apply to female flowers only. But anatomical examinations of infected female buds showed that there were no traces of mycelium in the ovary. The hypothesis would require that the mycelium is first formed in the stigmata, then by-passes the ovary and forms spores in the anthers.

The remarks about flower infection are also true to some extent for bud infection, although here the spores have a greater chance to germinate in the water trapped in the axils of leaves where the buds are.

Inspections of the infected area at Boarhills showed that infected plants always formed definite patches growing adjacent to healthy plants. This is a clear suggestion of seedling infection. Seedlings must have emerged from underneath female plants and spores from the neighbouring infected plants were carried by insects or wind and deposited on the one locality.



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seedlings. Such spores may also be deposited on the seedling from the died-down branches of the plant above it. Germination of spores on seedlings was favoured by the fact that the latter were living in a humid microclimate created by the presence of the mature plants above them. Inoculation experiments showed that seedling infection is much more certain and perhaps more natural than flower or bud infection.

Field and greenhouse observations revealed that a naturally infected plant produced infected flowers throughout its life. This fact no doubt has great ecological implications. An infected male plant produces only spores and no pollen grains, likewise an infected plant produces no seeds. The two plants are therefore virtually sterile. The expected result would be that in any locality, heavy infections should ultimately lead to the extermination of the species and its replacement by other competing plants.

In the review of sex ratios in Silene dioica, Löve (1940) refers to "preponderance of females over males" by which she meant that in any locality more female plants would occur than male plants. This is not in accordance with the theory that in dioecious species, male and female plants should be equally represented. Shull (1912) refers to Silene as not according well with this theory, but he does not give reasons for his assumption. In the survey of Silene dioica plants growing in the three areas near St. Andrews this alleged departure from the theoretically expected numbers was not found. Root-tip squashes from a large selection of seedlings revealed a ratio that was approximately 50:50, and using statistical methods, the difference between the expected ratios and what was actually arrived at, was found not to be significant. It was concluded that in Silene dioica there was no real difference in the number of female and male plants in any one locality. One explanation for female preponderance assumes two



different pollen grains, one with a Y- chromosome and another with an X- chromosome, with the latter having a bigger chance of survival (Page 23). It must be remembered here that when dealing with pollen grains, we are in fact dealing with millions of cells and for an ovule to be fertilized, only one pollen tube is required, and it is hard to imagine that pollen tubes from potential female producing pollen grains have better chances of reaching the ovules. The only possible point that one might put in favour of Löve's statements and conclusions is that her work was conducted in Sweden where the long summer days may have altered the sex expression in favour of femaleness in some of the plants she examined. But even that would not explain the small percentage (29.19) of male plants quoted by her from the work of Correns (Page 24).

Silene dioica is not an economically important plant. But, according to mycological accounts, it has distinct male and female plants. This feature, which is rare in higher plants, has attracted the attention of many workers. But is sex in this species really stable? Shull (1910-1912) has carried out a number of experiments on sex reversals and referred to natural hermaphrodites in this species. Other workers e.g. Löve (1940) refer to intersexes and other polyploids exhibiting various degrees of maleness, femaleness and hermaphroditism.

In the present work two plants have been described each having the normal diploid male chromosome complement, but in every flowering branch, the leading blossom was female. The phenomenon could not be explained by cytological or genetical means. The progeny of these two plants have not so far shown the same phenomenon. It is believed that what might have happened here is that the environment in the greenhouse may have shifted the sex of these leading flowers from maleness to femaleness.

Sex in Silene dioica judging from the foregoing statements is by no means stable. Environmental factors can cause a shift. Attacks by U. violacea provide evidence that what appear to be distinct male



ions. The primary infections, whether through the flower, soil, or and female flowers are in fact essentially hermaphroditic, a fact which has been confirmed by anatomical investigations.

Germination experiments of chlamydospores showed that there was no rest period and that they germinated, when fresh, after just over 4 hours at 20°C. Storage and survival experiments revealed that such spores were still viable and showed a high percentage of germination after storage under various conditions for 24 months. Another point is that chlamydospores have a wide range of temperature for germination (5-38°C). These facts would imply that chlamydospores are able to overwinter in the soil or in parts of the infected plants. When such plants die down in the winter, chlamydospores remain adhering to the died-down parts and in the next spring when new shoots come out, these spores may attack them and thus perpetuate the infection from one season to another. Baar (1903) claims to have found the mycelium overwintering in the rootstocks. Other workers e.g. Baker (1947) simply referred to the mycelium as being systemic and overwintering in the rootstocks. Others, reported by Fischer (1957), deduced this condition of the mycelium from the fact that once a plant is infected it would continue to be so for the rest of its life. The logic of the argument given here cannot be denied, but since all attempts to find the mycelium in the rootstocks failed, perennation there, as advocated by Baar needs to be questioned. One other way to explain the fact that infected plants show such infections every season, is to suggest that fresh spores from died-down branches land on the new branches and attack the axillary buds.

It has often been found, especially after a rainfall, that the spores collected were at various stages of germination within the flower. Germinated spores were also observed on other parts of the infected plant. These findings led me to believe that an infected plant may have become so as a result of primary and secondary infect-



ions. The primary infections, whether through the flower, bud, or seedling give rise to the first infected flowers or first infected branches. Other branches should not necessarily get the infection through invasion by the same mycelium but fresh spores from the same plant may cause infections to other branches.

When chlamydospores are germinated in nutrient media, promycelia of three and very rarely four cells emerge from the spores. These promycelia bud sporidia from their three or four cells. The sporidia in turn bud secondary sporidia and the process is repeated indefinitely or as long as the nutrients are not depleted. Now, when such germination take place in water, sporidia soon fuse in pairs. In interpreting these fusions, the earlier workers, notably de Bary and Brefeld, could not agree as to whether such fusions were sexual or simply somatic. The argument put forward by de Bary was that in such fusions only two cells took part whereas if it were somatic, he could not see what would stop a third cell from joining the fused pair. Brefeld, on the other hand, argued that if such fusions were to be considered as sexual, then the pairing cells ought to be looked upon as gametes. But continuous budding of the sporidia in nutrient media makes them different from conventional gametes, which do not undergo any growth before fertilization.

To reconcile these two arguments, one ought to look at what happens to the nuclei when chlamydospores germinate. At the onset of germination, the spore nucleus divides (Fig. I Plate XVI). Four chromosomes are observed. In the meantime the spore wall bulges to one side and a germ tube emerges. The spore nucleus then divides into two and one of the daughter nuclei migrates into the germ tube and the other remains in the spore. A similar division takes place in the germ tube and the two cells formed each have one nucleus with two chromosomes (Fig. II Plate XVII). This is the haploid number of chromosomes which should logically be the number in the sporidia, since they are budded off vegetatively from the promycelial cells. It was noticed in many instances that fusions between sporidia and promycelial cells did occur, which confirms that they



must have the same chromosome complements. When two sporidia fuse, the nuclei in them do not migrate but remain in their respective cells. This means that the initial stage in the fusion is merely somatic. But the fact remains that only two haploid sporidia can form such a union. This means that sporidia must be of two different mating types or potentially they represent two different sexes. Within the host plant, the nuclei from the two sporidia remain associated but distinct throughout the parasitic life and even after the formation of the young spores. Fusion or caryogamy is therefore deferred until the maturation of the chlamydospores. The controversy referred to earlier need not to have arisen at all if we bear in mind that the ultimate result of this somatic fusion of sporidia is fertilization in the chlamydospore where the nuclei one coming from each sporidium fuse to form the diploid nucleus in the mature chlamydospore.

The life history of Ustilago violacea is an example of the haplobiontic-diplobiontic cycle in which the vegetative haploid sporidial mycelium gives rise to the dikaryotic parasitic mycelium through somatogamous fusion of sporidia. The dikaryotic parasitic mycelium produces the diploid resting spores.

Mycologists have for some time adopted the term chlamydospores to describe the resting spores of the smut fungi. Other mycologists used the term teleutospores which is a reminder of the supposedly close relationship between smuts and rusts. The term chlamydospore as used for the smut fungi is misleading, because true chlamydospores which are vegetative resting cells, functioning as reproductive bodies as in Fusarium udum and Phytophthora species, are asexual and intercalarily formed. In U. violacea and perhaps in a large number of other smuts, the resting spores are formed as a result of the breaking up of the dikaryotic mycelium into smaller units each with two nuclei which later on fuse to form the diploid nucleus in the mature spore. Therefore the resting spore is (a) diploid and (b) sexually formed. Thus it might be said that the smut resting spores are analogons with the



teleutospores of the rust fungi. This is why the more recent publications by Bessey (1950) and Alexopoulos (1962), refer to them as teleutospores or simply teliospores.

The term promycelium given to describe the germ tube arising from smut spores is more appropriate than the former name hemi-basidium although the latter name links the smuts with the Basidiomycetes. But developmentally the germ tube of the smuts is different from the basidium in higher Basidiomycetes. In the Basidiomycetes, reduction division occurs within the basidium to give rise to the basiospores. But in the smuts, reduction division occurs in the chlamydospores and the promycelial cells are therefore haploid. Gäumann (1962) reports that meiosis occurs in the promycelial cells in smuts. This point is believed by the author to be inaccurate. Apart from what is current in the literature, results obtained from stained preparations confirmed that reduction division of the chlamydospore occurred within the spore and only the second meiotic division was in the first promycelial cell.

- ii. The history, classification and nomenclature of *Ustilago* are reviewed, especially because of the fact that the fungus attacks a large number of host plants and has been described under different names. It is under different names, depending on the host plant on which it was discovered. The history of the fungus is traced back to the first recorded occurrence of the fungus on wheat in 1829. The life history of the fungus is reviewed. The occurrence of the fungus is recorded in 1829.
- iii. Field observations in the vicinity of St. Albans, Herts.

out to find the natural proportions of the two sexes and whether there were any hermaphrodites. Three areas were selected and an average of 50 plants in each area were recorded. Male plants were found to be only slightly in excess of females - the ratios were 57:100. This field work was supplemented by results taken from plants growing in the greenhouse. Root-tips squashed at known definite numbers of anthers were also used to estimate the sex ratio. Series of infected plants were also determined by the root

#### SUMMARY

- i. The history of the species Silene dioica was briefly reviewed. Special emphasis was laid on the taxonomic position which was not certain up to 10 years ago; different writers spoke of the species as Lychnis dioica, Melandrium rubrum or Silene dioica and they were all referring to the same species commonly known as the Red campion. Chawdhuri's revision of the genus Silene resulted in placing the red campion and other allied plants in the genus Silene. His system of classification is now adopted in all the recent works. Publications relating to the distribution and sexuality of the species were also reviewed. Sex in Silene dioica is controlled by the X:Y ratio where X represents the female and Y the male sex chromosome.
- ii. The history, classification and nomenclature of Ustilago violacea are reviewed, especially because of the fact that the fungus attacks a large number of host plants and some of the earlier workers referred to it under different names dependant on the host plant in which it was discovered. The secondary effects of induced hermaphroditism on the host plants and the different versions of the life history of the fungus are reviewed. References to the occurrence of the disease in Britain are given.
- iii. Field observations in the vicinity of St. Andrews were carried



out to find the natural proportions of the two sexes and whether there were any hermaphrodites. Three areas were selected and an average of 80 plants in each area were recorded. Male plants were found to be only slightly in excess of females - the ratios were 52:48. This field work was supplemented by results taken from plants growing in the greenhouse. Root-tips squashes of some definite numbers of seedlings were also used to estimate the sex ratios. Sexes of infected plants were also determined by the root squash methods.

- iv. Seed transmission of the disease was investigated. Seeds were collected from infected areas and examined for chlamydospores on their coats - results were negative and the plants raised from such seeds were healthy. The surfaces of seeds then contaminated with spores and sown. The plants raised were also healthy. Seed transmission of the disease was therefore excluded.

Inoculation experiments were performed on adult plants as well as seedlings. In the case of adult plants, flower and bud inoculations were tried and also inoculations under the epidermis in the younger parts of the stem and the young leaves. Seedlings were either inoculated in the terminal buds or soaked in spore suspensions.

- vii. The results obtained showed a high percentage of infections in bud inoculations, slightly less infections in flower inoculations and very little percentage in leaf or stem inoculations. Very high infection rates were caused by seedling inoculations. Seedlings were grown in spore infested soils and practically no infection resulted.

v. The nuclear behaviour of spores was examined. Various fixatives and stains were employed and Azure "A" preceded by Newcomers' fixative gave the best results. Young and mature chlamydospores were fixed and stained. Two nuclei were observed in the young spores and only one was observed in the mature ones. In spores which were fixed at the point of germination, dividing nuclei were observed in the chlamydospores and in the promycelial cells. The diploid number of chromosomes was four. Observations on conjugating sporidia were made - fusions were considered to be only somatic since no migrations of the nuclei took place.

vi. Spore germination experiments were performed at different temperatures and pH. values. The optimum temperature was in the range 16-22°C, the maximum was 38°C and the lowest was 5°C. Different concentrations of glucose and malt extract were also used. The optimum pH. for germination at 21°C was pH. 6.6.

Different concentrations of glucose and malt extract were used as media for sporidial fusions at 19°C and 25°C. Results indicated that fusions were inversely proportional to concentration and were favoured by the higher temperature.

Storage and survival experiments showed that spores were viable after 24 months and that fresh spores gave higher rates of germination than stored ones.

vii. The mycelium in infected plants was traced through a series of microtome sections made from different parts of the plant. Hyphae were detected in the younger parts of the stem especially at the nodes, near the growing points and in the flower buds. In the stem, hyphae were found close to and parallel to the vascular system. On older infected anthers, the plant tissue were completely replaced by spores.



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Experiments were performed where the anthers from male flower buds and ovaries from female flower buds were removed. The buds shrivelled up as a result of the operation.

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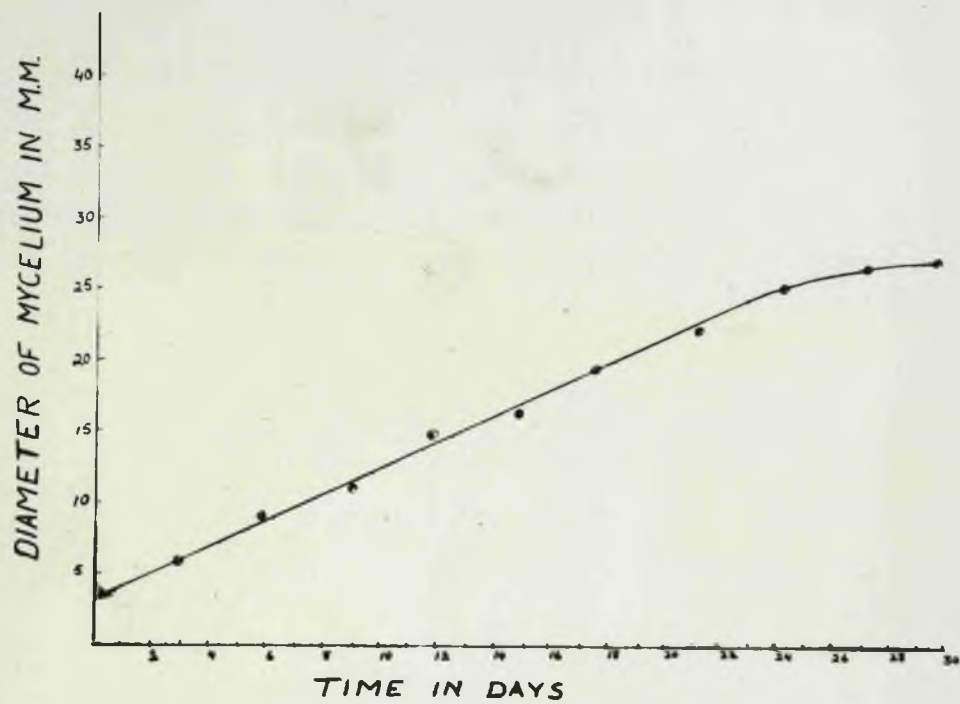


PLATE II

Fig. I.

Photograph showing the general appearance  
of healthy female flowers and buds.

X 3.

Fig. II.

Photograph showing the general appearance  
of healthy male flowers and buds.

X 3.



I



II



## PLATE II

Fig. I.

Photograph showing the general appearance  
of healthy female flowers and buds.

X 3.

Fig. II.

Photograph showing the general appearance  
of healthy male flowers and buds.

X 3.





PLATE III

Fig. I.

Photograph of an infected female flower.

X 3.

Fig. II.

Photograph of an infected male flower.

X 3.





## PLATE III

Fig. I.

Photograph of an infected female flower.

x 3.

Fig. II.

Photograph of an infected male flower.

x 3.





PLATE IV.

Fig. I.

Graph showing the rate of germination of Chlamydospores in different phosphate buffer solutions with different pH. values. Plotted as percentage germination against pH. at 21°C.





Fig. I.

Graph showing the rate of germination of Chlamydozoospores in different phosphate buffer solutions with different pH. values. Plotted as percentage germination against pH. at 21°C.



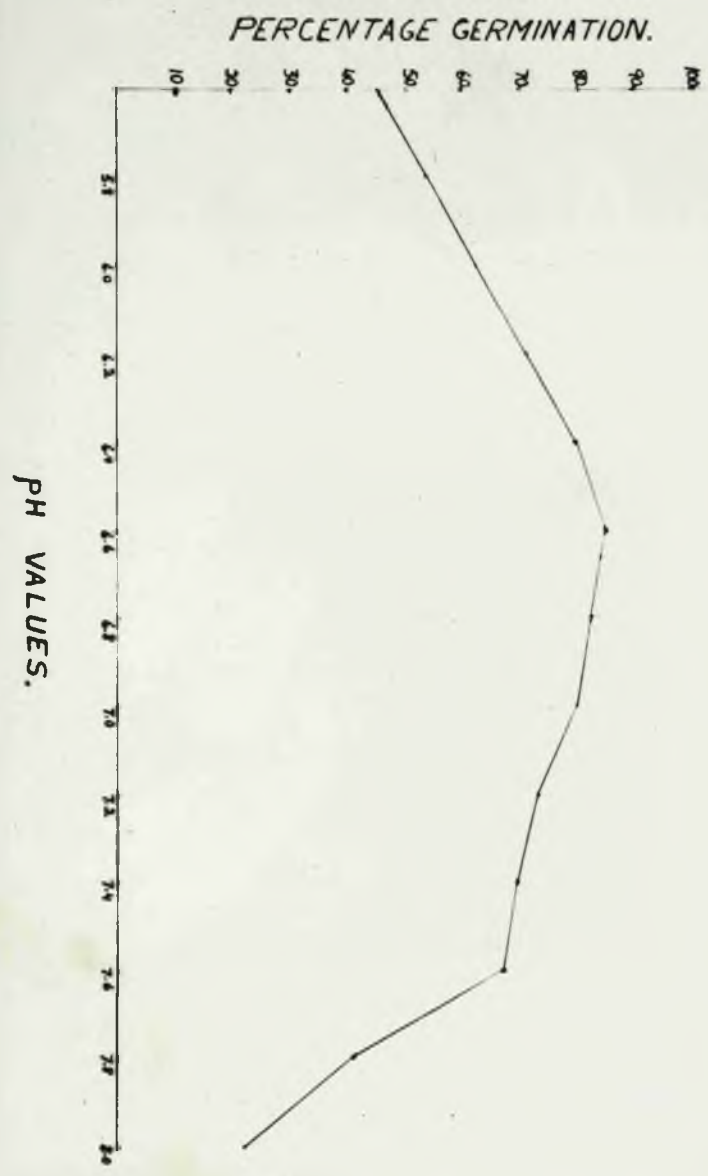


PLATE V.

Fig. I.

One of the unusual promycelia with 4 cells.  
A sporidium is still attached to the distal cell.

Fig. II.

A germinated chlamydospore with a three-  
celled promycelium.



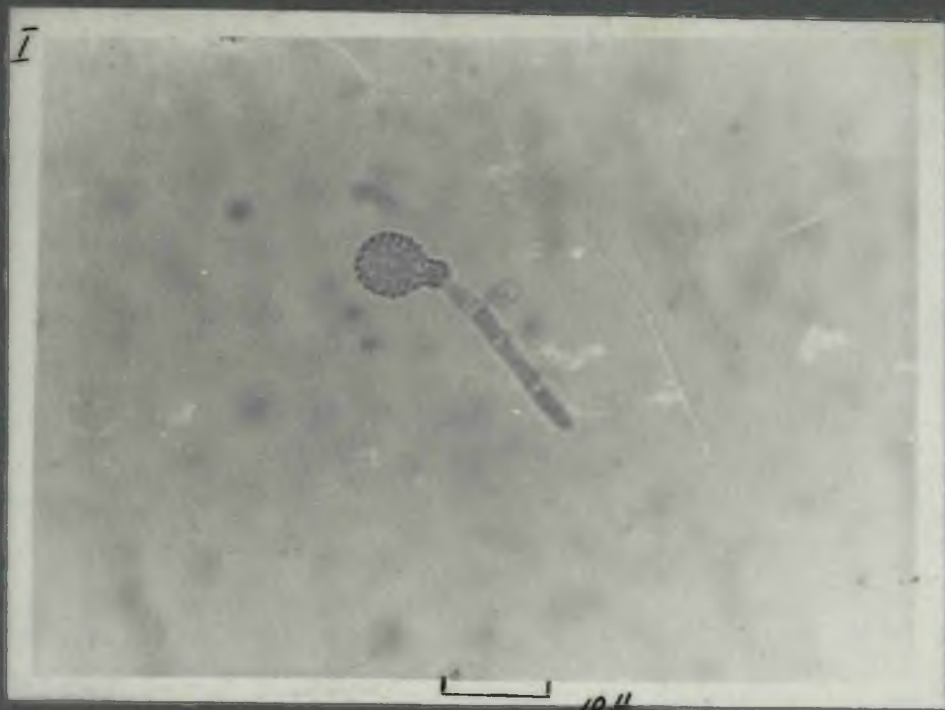


Fig. I.

One of the unusual promycelia with 4 cells.  
A sporidium is still attached to the distal cell.

401



Fig. II.

A germinated chlamydospore with a three-  
celled promycelium.

42







PLATE VI.

Fig. I.

a) Germinated chlamydospore showing two promycelia coming off two points.

b) A promycelium just detached from the spore.

Fig. II.

Chlamydospore showing two promycelia of different ages arising from the same point.



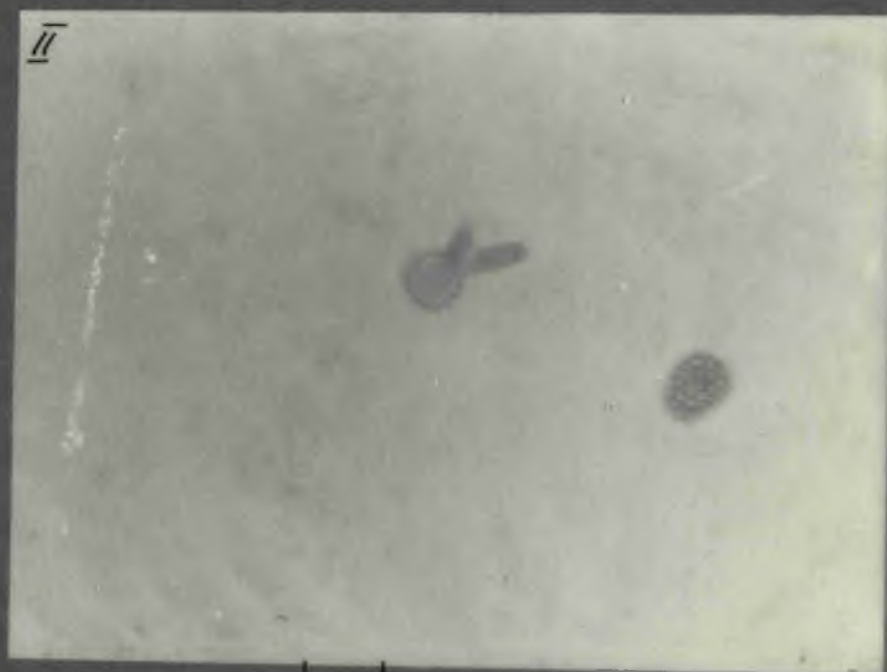
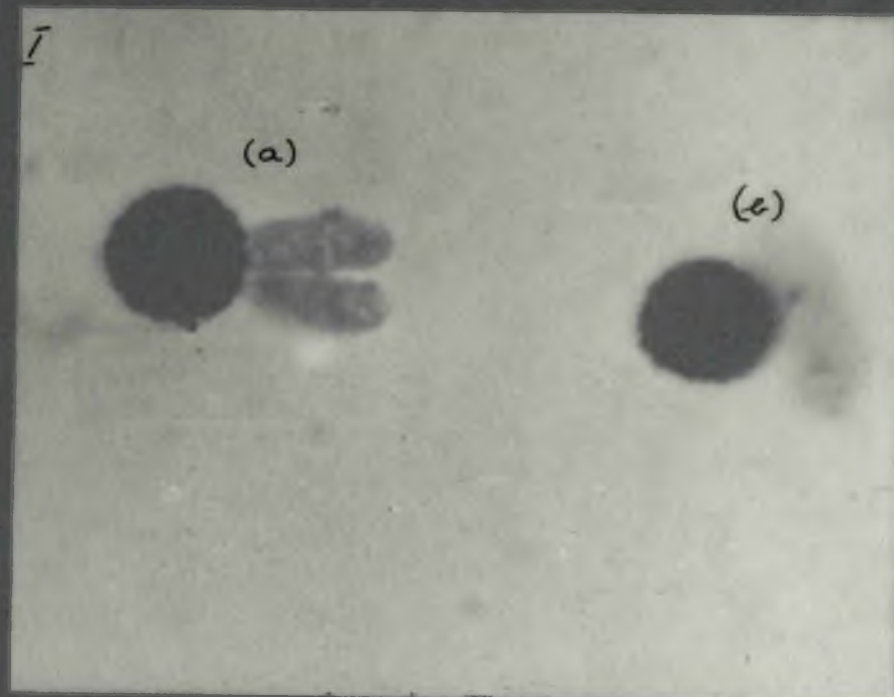


PLATE VI.

1

(5)

Fig. I. (3)

a) Germinated chlamydospore showing two promycelia coming off two points.

b) A promycelium just detached from the spore.

1/2

Fig. II.

Chlamydospore showing two promycelia of different ages arising from the same point.

1/4



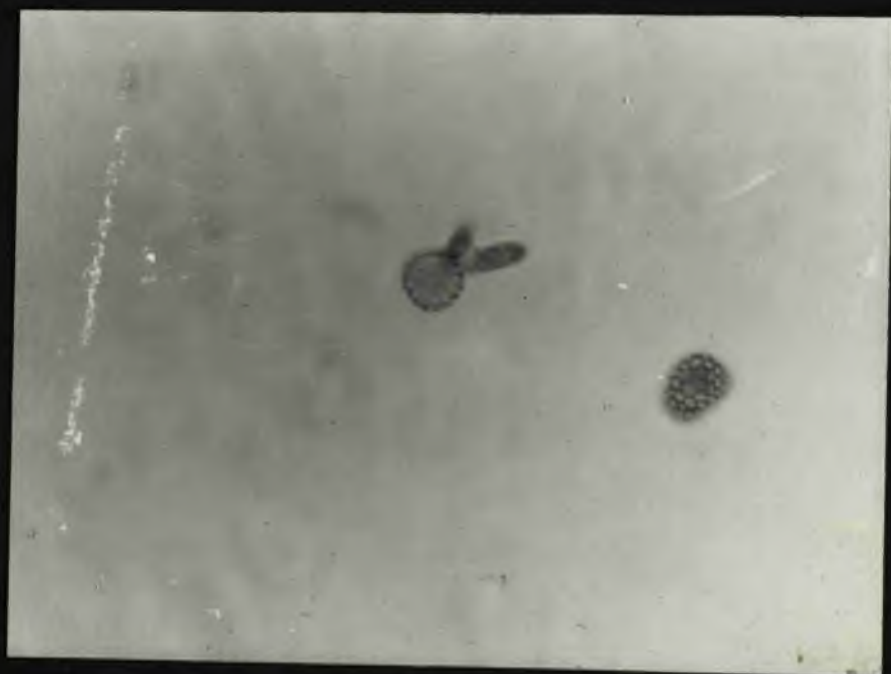
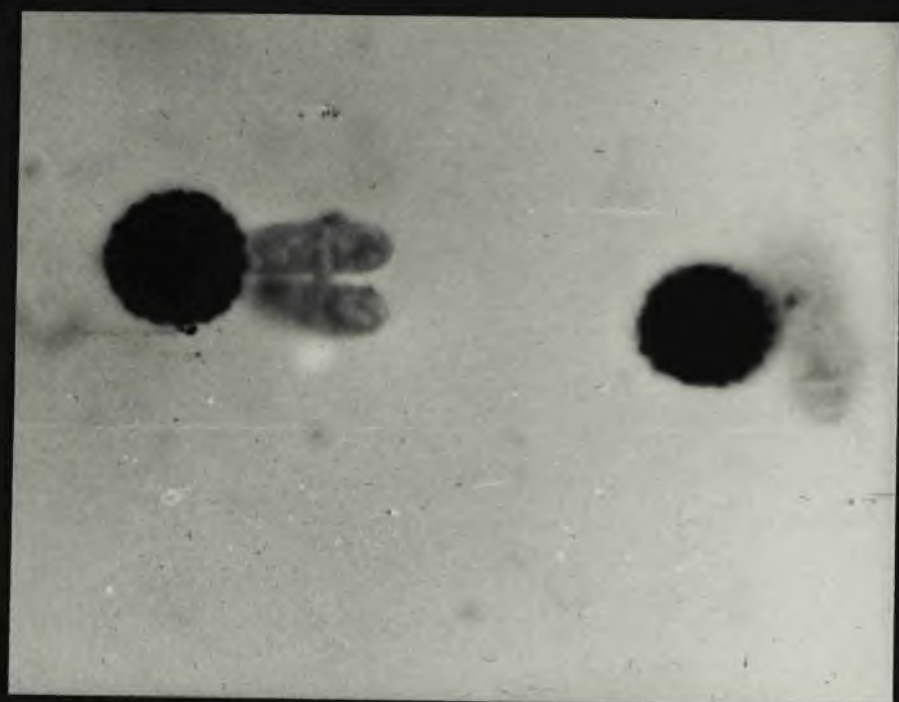


PLATE VII.

Fig. I.

Two sporidia fusing end to end . A small swelling in the conjugation tube is noticeable.

Fig. II.

A sporidium fusing with a promycelial cell.

Fig. III.

Two sporidia fusing through tubes arising centrally.



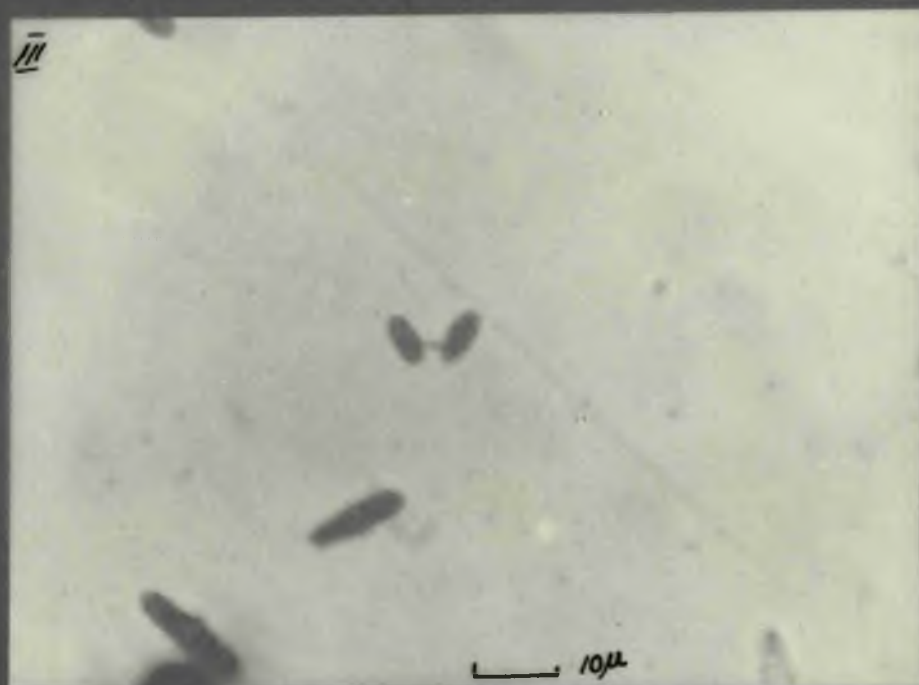


PLATE VII.

1

Fig. I.

Two sporidia fusing end to end. A small swelling in the conjugation tube is noticeable.

Fig. II.

A sporidium fusing with a promycelial cell.

42

42

Fig. III.

Two sporidia fusing through tubes arising centrally.

401



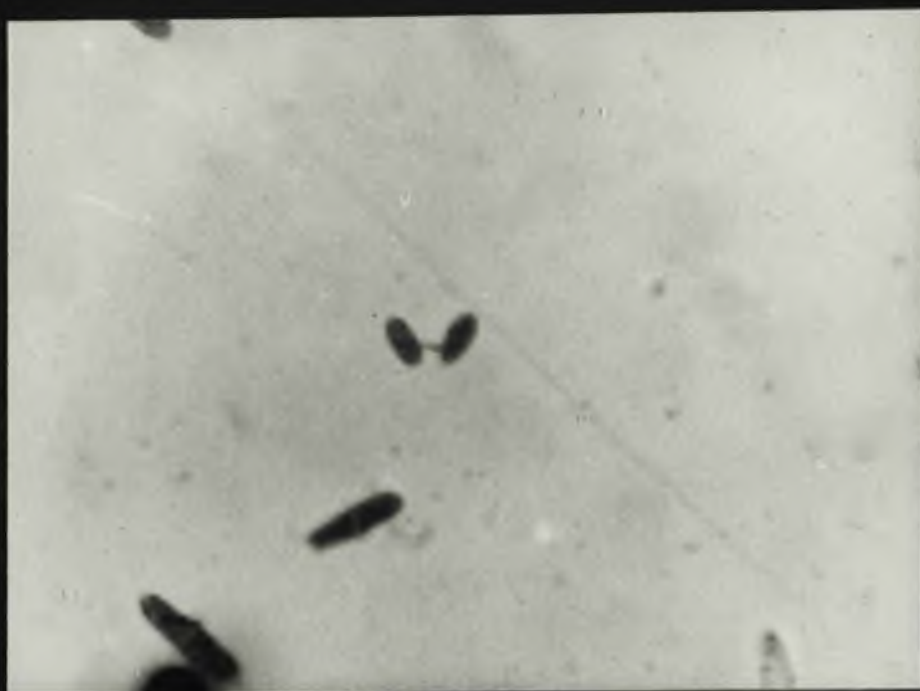


PLATE VIII

Fig. I.

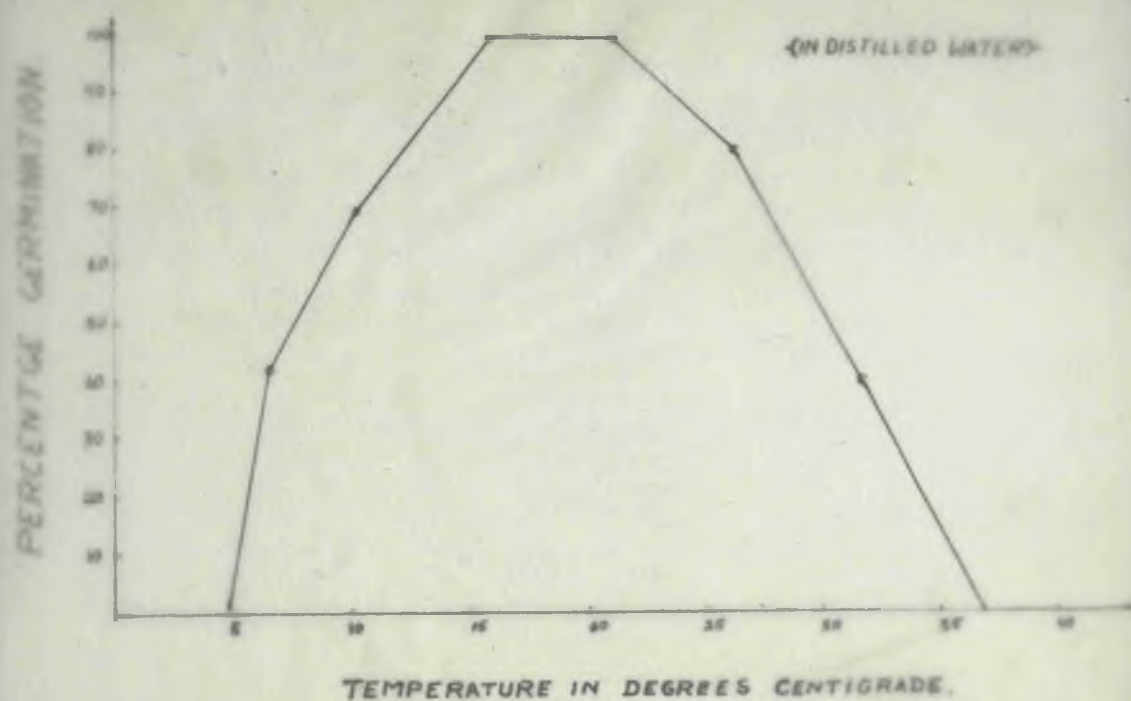
Graph showing the rate of germination of chlamydospores in distilled water at different temperatures. Plotted as percentage germination against temperature in °C.

Fig. II.

Graph showing the rate of germination of chlamydospores in 1% malt extract. Plotted as percentage germination against temperature in °C.



I



II

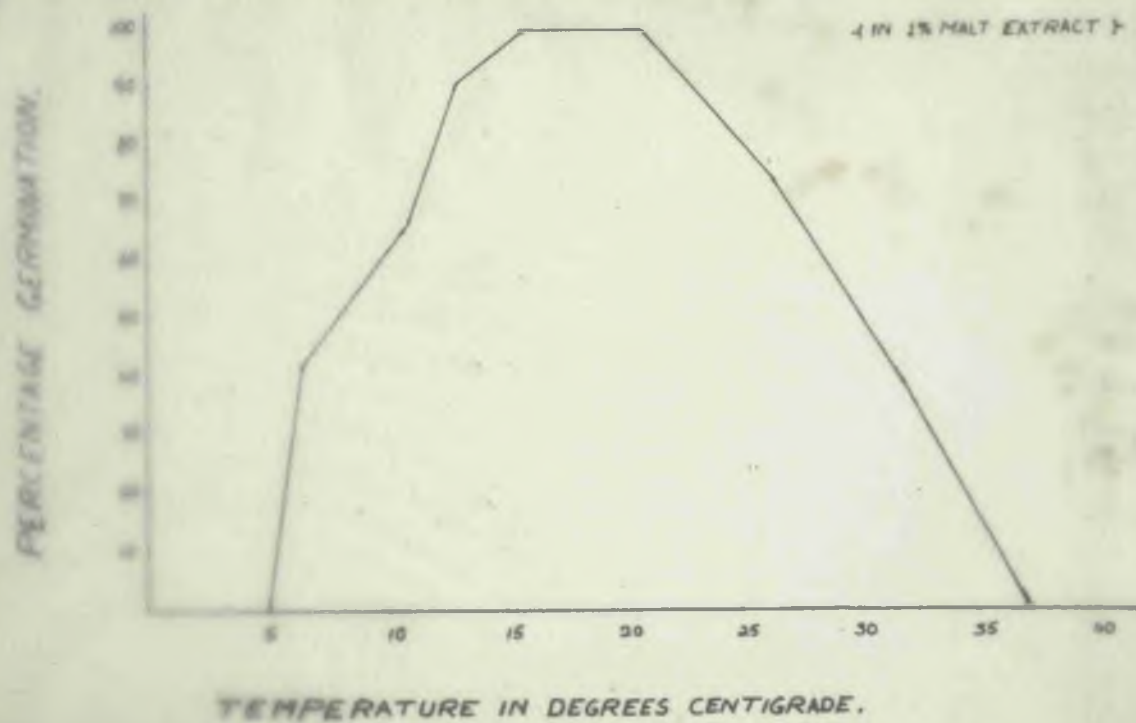


Fig. I.

Graph showing the rate of germination of chlamydospores in distilled water at different temperatures. Plotted as percentage germination against temperature in °C.

Fig. II.

Graph showing the rate of germination of chlamydospores in 1% malt extract. Plotted as percentage germination against temperature in °C.



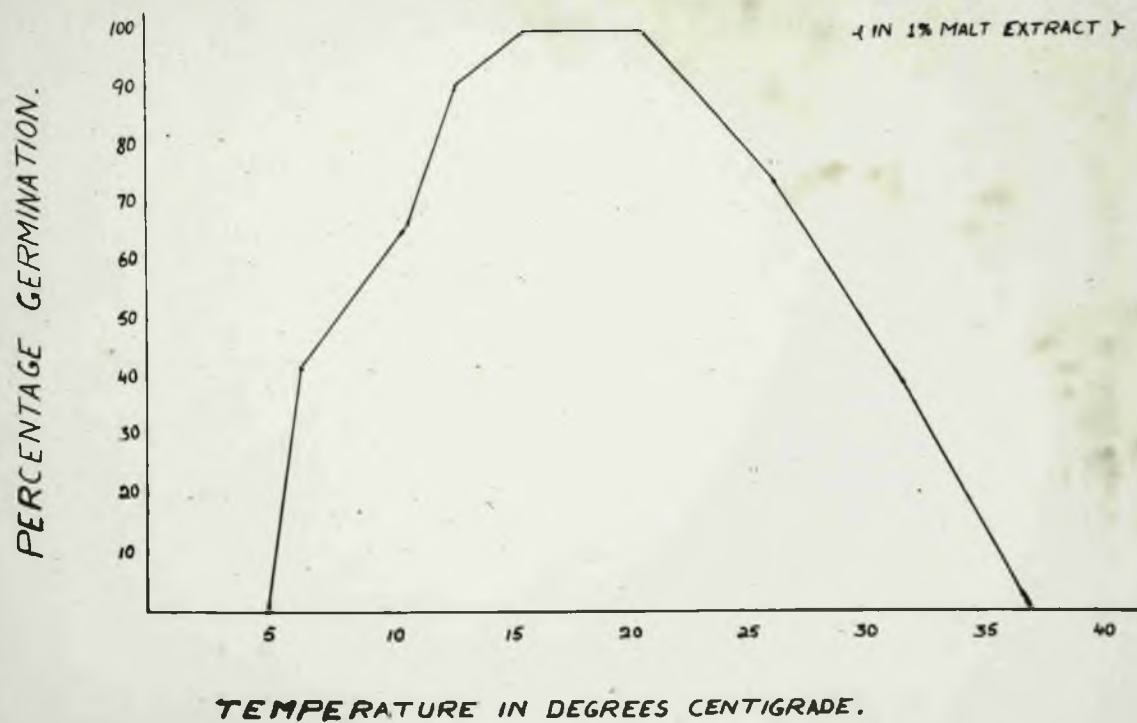
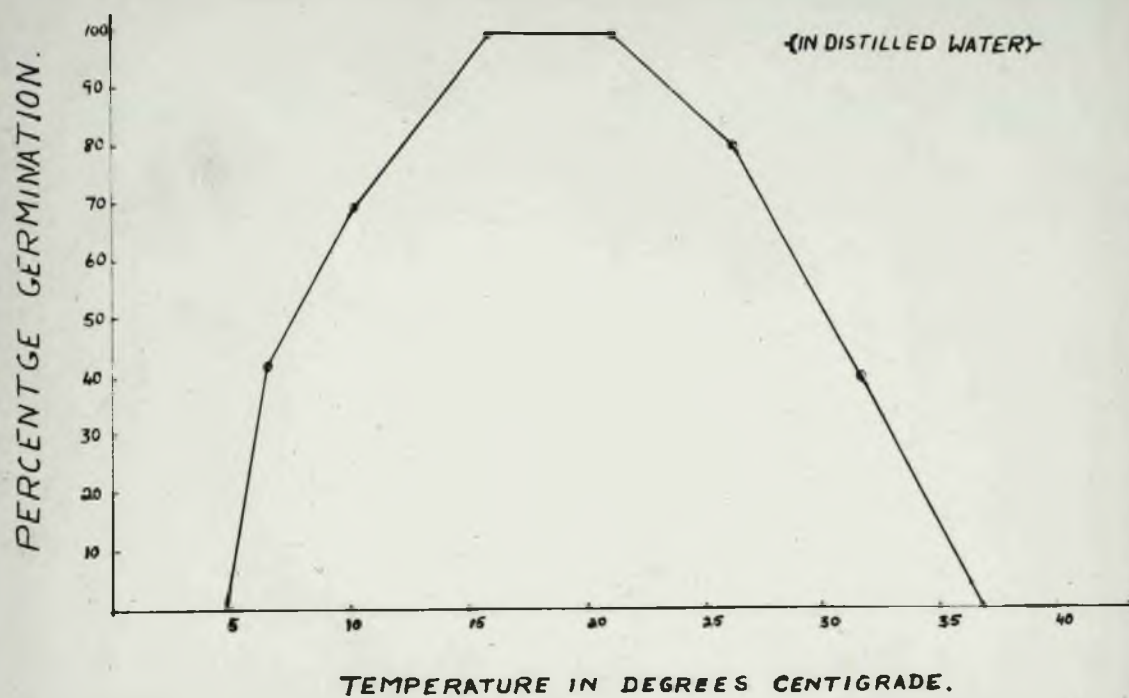


PLATE IX

Fig. I.

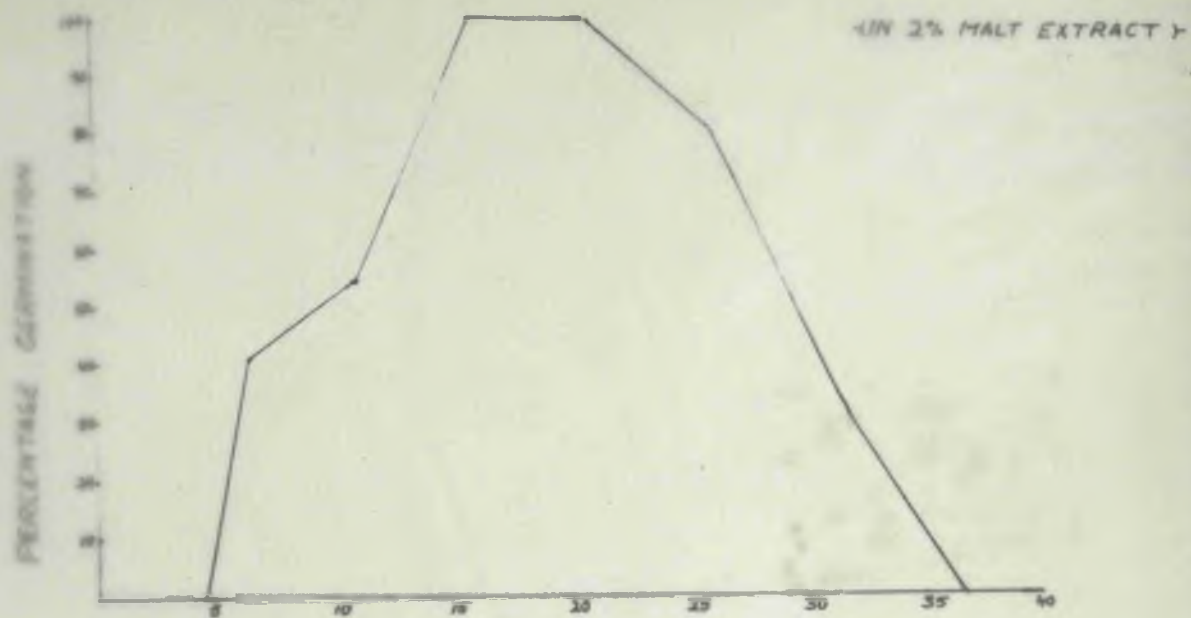
Graph showing the rate of germination of chlamydospores in 2% malt extract at different temperatures. Plotted as the percentage germination against temperature in °C.

Fig. II.

Graph showing the rate of germination of chlamydospores in 5% malt extract at different temperatures. Plotted as the percentage germination against temperatures in °C.

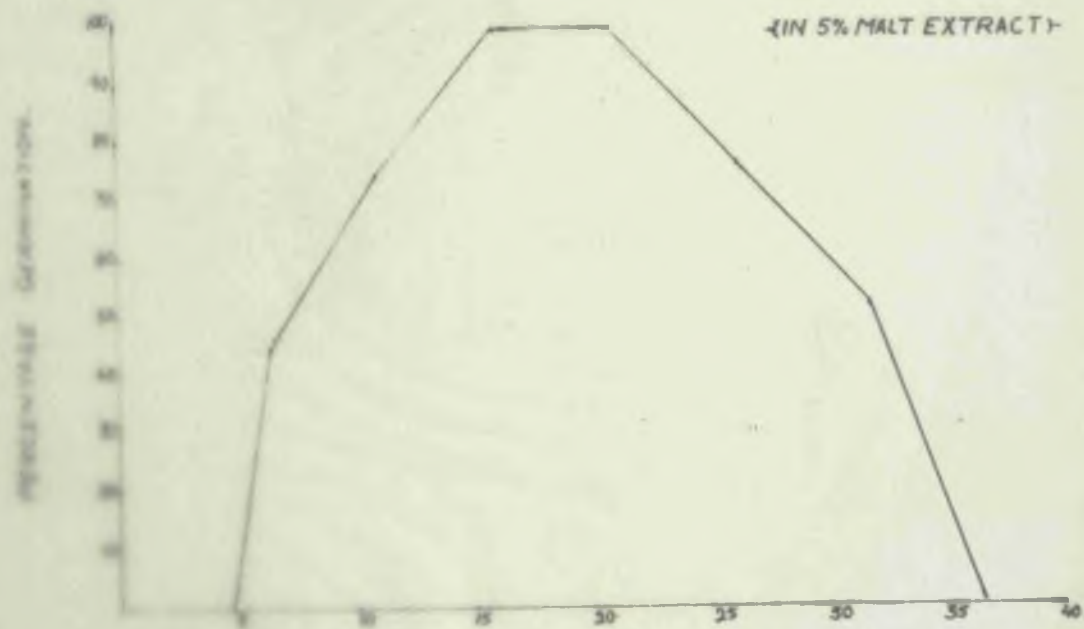


I



TEMPERATURE IN DEGREES CENTIGRADE.

II



TEMPERATURE IN DEGREES CENTIGRADE.

## PLATE IX

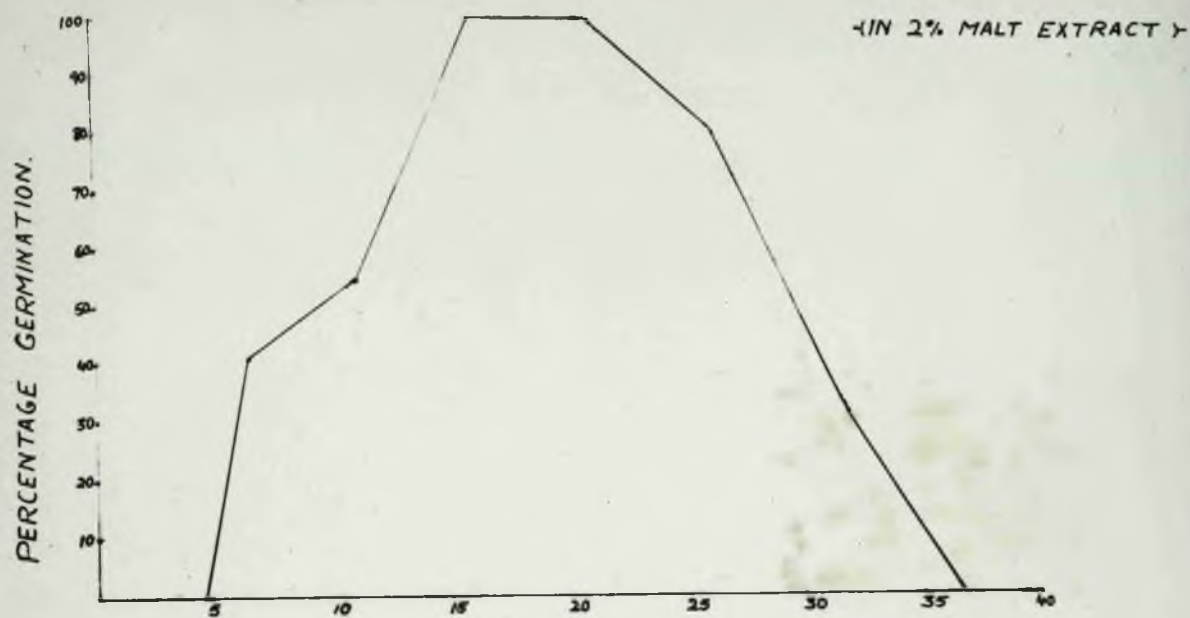
Fig. I.

Graph showing the rate of germination of chlamydo-spores in 2% malt extract at different temperatures. Plotted as the percentage germination against temperature in °C.

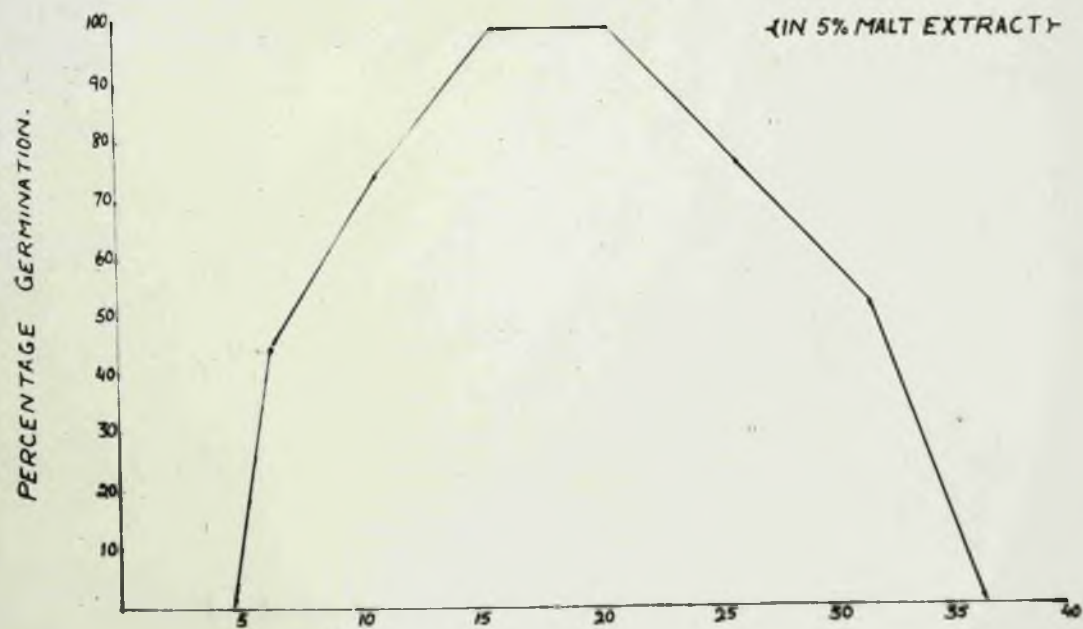
Fig. II.

Graph showing the rate of germination of chlamydo-spores in 5% malt extract at different temperatures. Plotted as the percentage germination against temperatures in °C.





TEMPERATURE IN DEGREES CENTIGRADE.



TEMPERATURE IN DEGREES CENTIGRADE.

PLATE X

Fig. I.

An infected male flower dissected to show  
the induced ovary. x3

Fig. II.

A longitudinal section through a bud from  
an infected male plant showing spores and  
mycelium in the anthers. Mycelium is also  
shown at the base of the ovary. (↗)



I



II

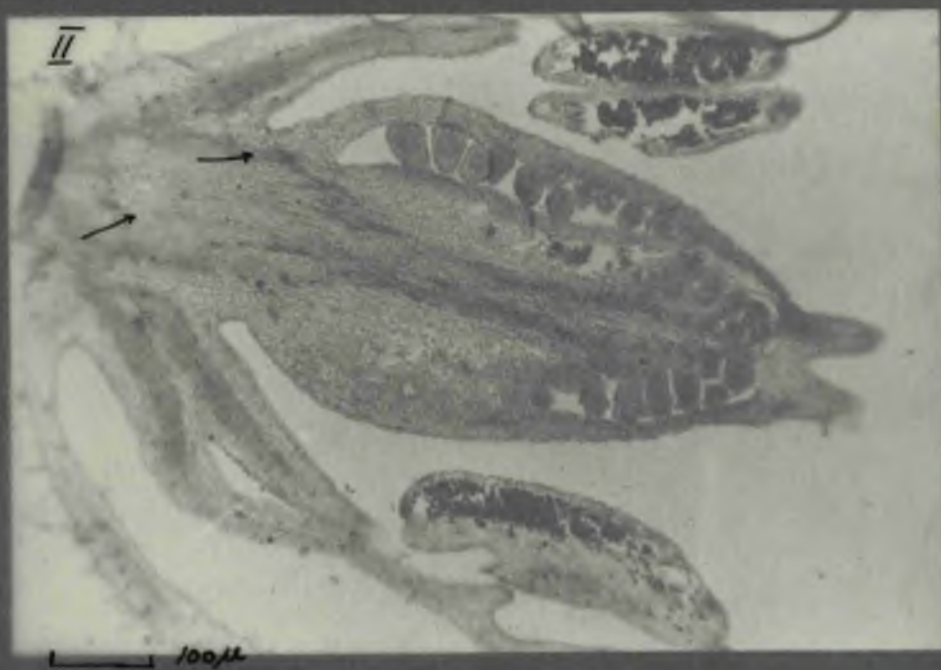


Fig. I.

An infected male flower dissected to show  
the induced ovary. x5

Fig. II.

A longitudinal section through a bud from  
an infected male plant showing spores and  
mycelium in the anthers. Mycelium III also  
shown at the base of the ovary. (↗)





PLATE XI

Fig. I.

Longitudinal section of a bud from an infected female plant showing spores and mycelium convolutions in the induced anthers. Pathes of mycelium are also seen at the base of the ovary. (→)

Fig. II.

Longitudinal section of an anther full of mature spores. The tapetal cells have already disappeared.



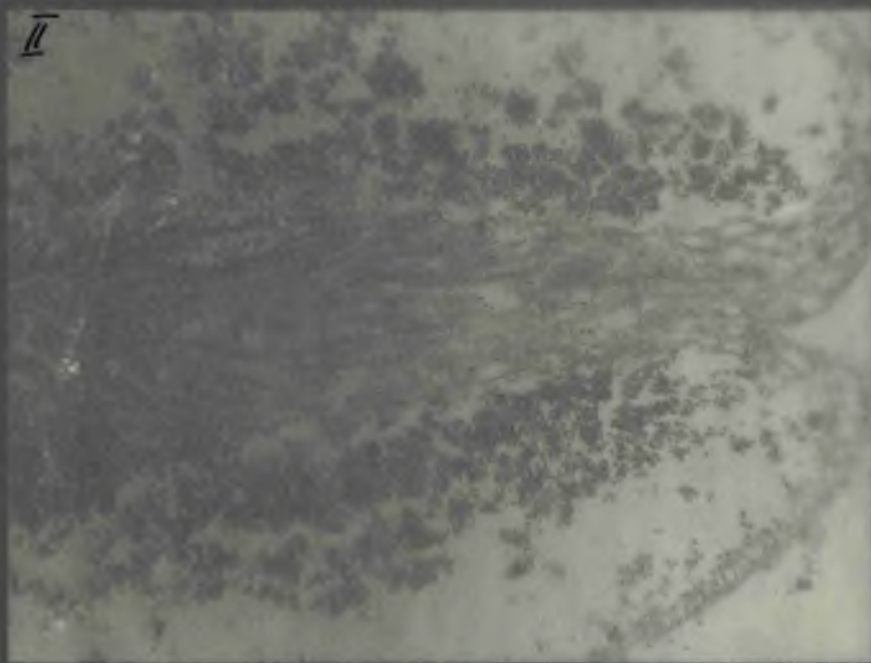
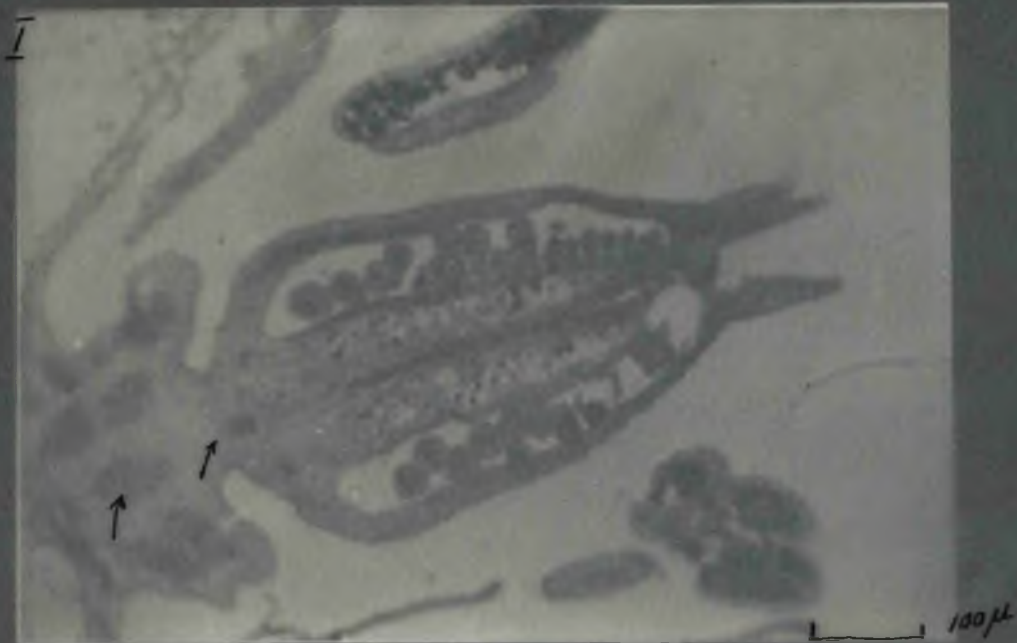



Fig. I.

Longitudinal section of a bud from an infected female plant showing spores and mycelium convolutions in the induced anthers. Patches of mycelium are also seen at the base of the ovary. (→)

4001 

Fig. II.

Longitudinal section of an anther full of mature spores. The tapetal cells II have already disappeared.

422 



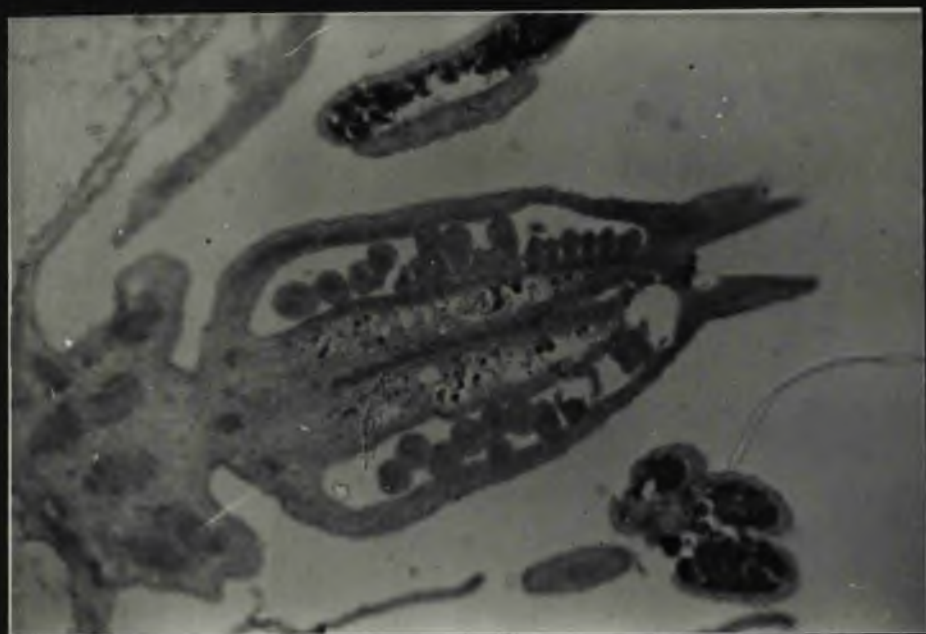


PLATE XII

Fig. I.

Longitudinal section through a pedicel  
showing a hypha in the intercellular space.  
( Bouin's fixative & Heidenhain's haematoxylin  
stain. )

Fig. II.

Longitudinal section of stem two nodes below  
the growing point, showing hyphae in the xylem  
parenchyma. ( Helly's fixative and Magdala  
red / Fast green. )

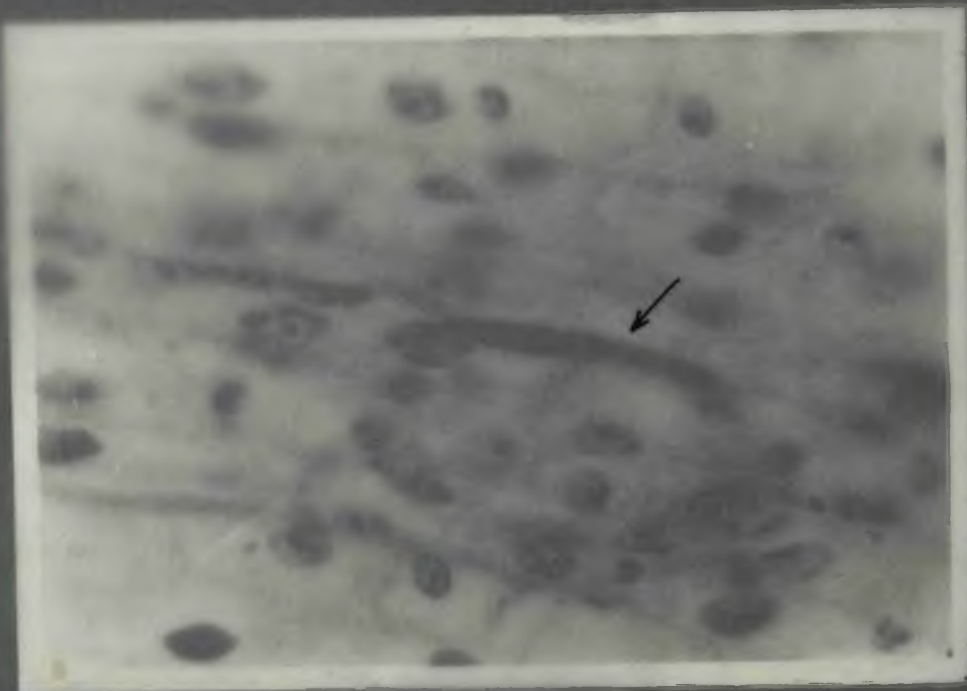


10 $\mu$



II

10 $\mu$



I

Fig. I.

Longitudinal section through a pedicel  
showing a hypha in the intercellular space.  
( Bouin's fixative & Heidenhain's haematoxylin  
stain. )

401 [ ]

Fig. II.

Longitudinal section of stem two nodes below  
the growing point, showing hyphae in the xylem  
parenchyma. ( Kelly's fixative and Magdala  
red / Fast green. )

↑ ↑

401 [ ]



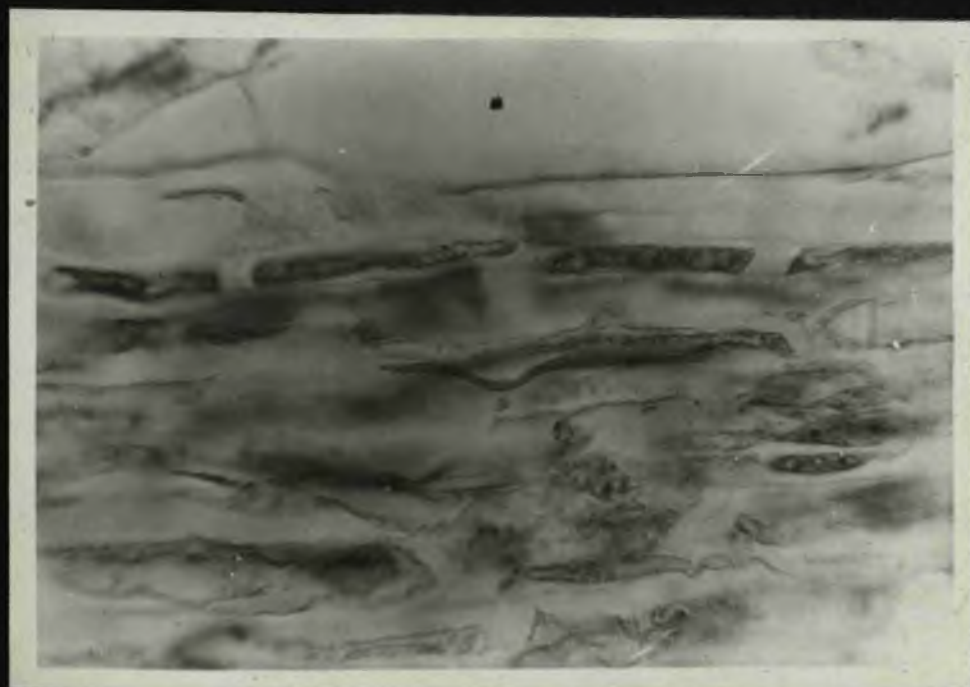
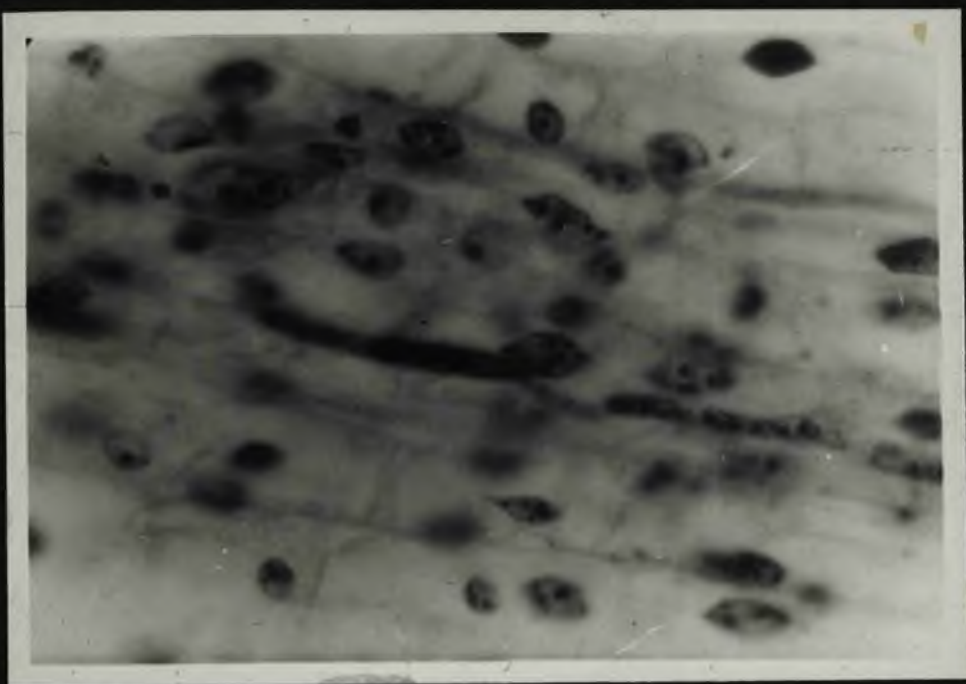


PLATE XIII

Fig. I.

Young spores (↗) showing two nuclei.  
( Newcomer's fixative & Azure A. )

Fig. II.

Mature spores showing single nuclei (→)  
and large central vacuoles (\*↗).



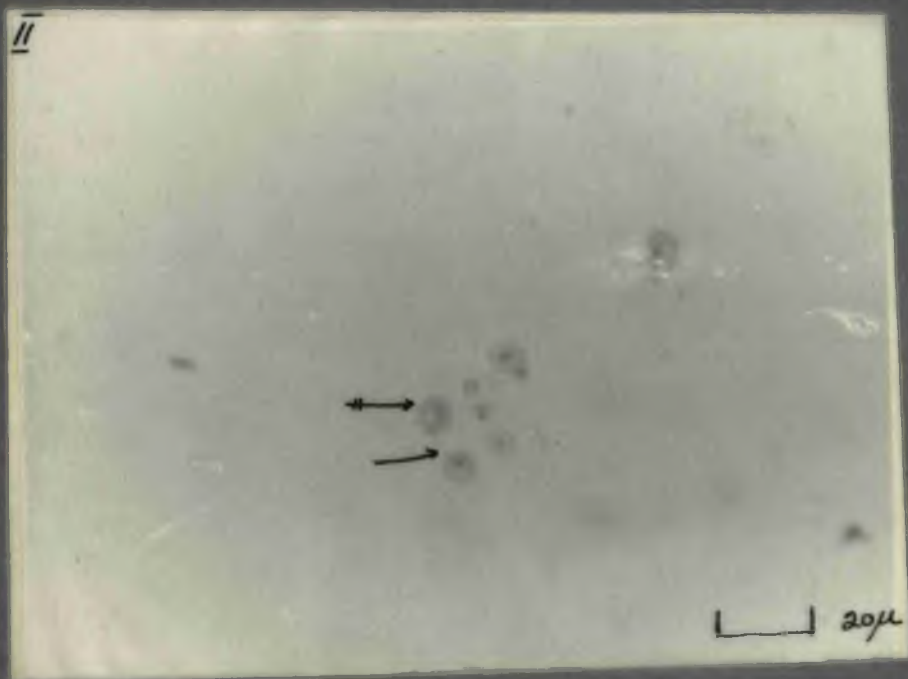
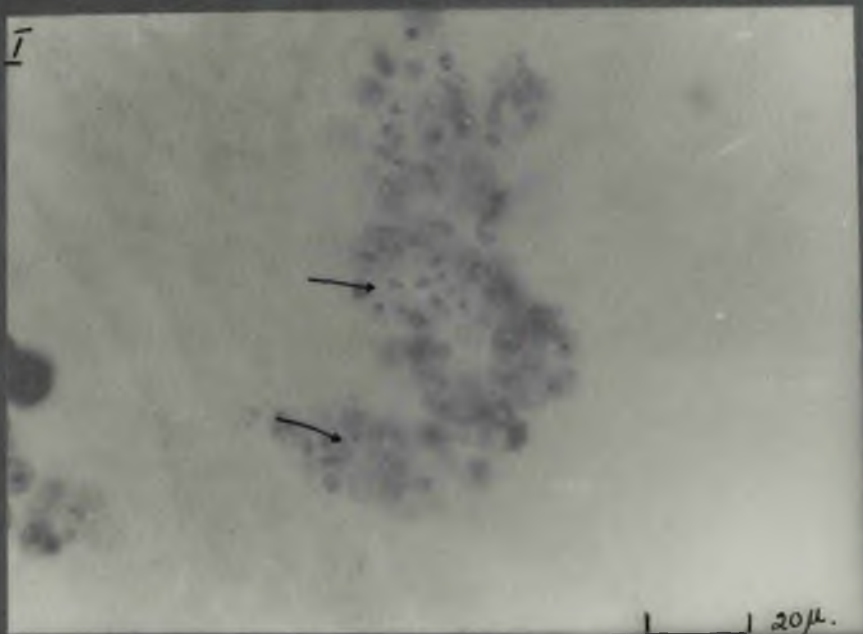


Fig. I.

Young spores (↗) showing two nuclei.  
(Newcomer's fixative & Azure A.)

100 μ

Fig. II.

Mature spores showing single nuclei (→) and large central vacuoles (↗).

100 μ

100 μ



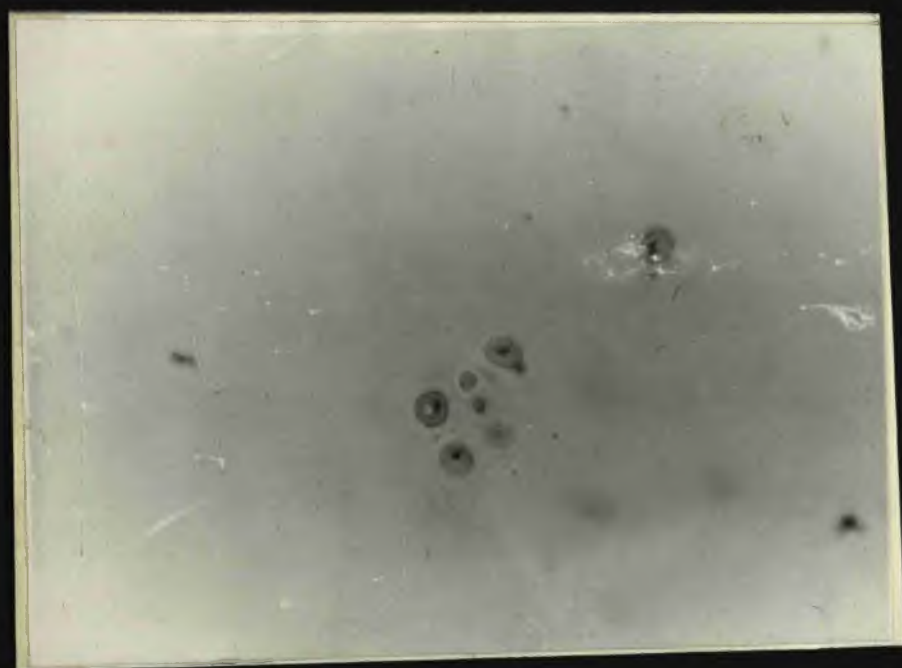
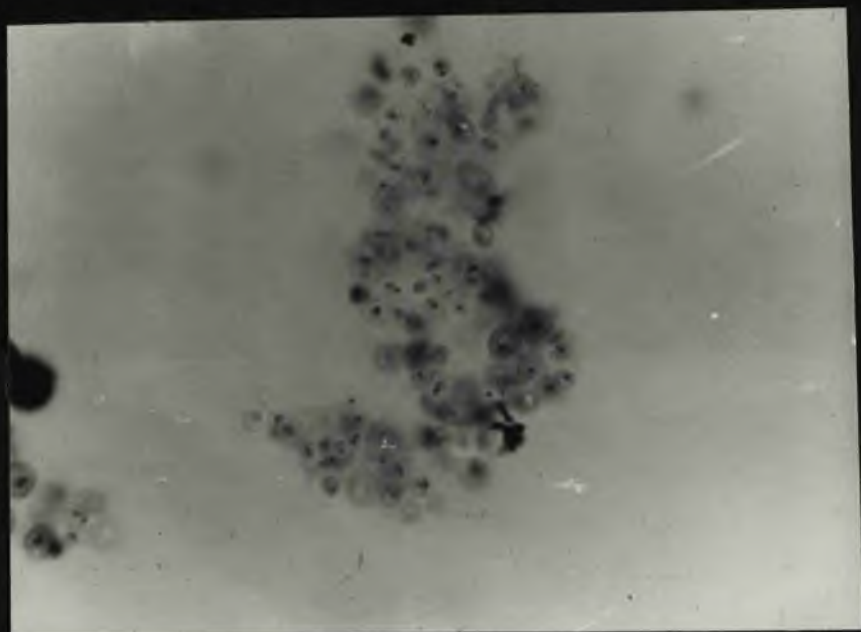


PLATE XIV

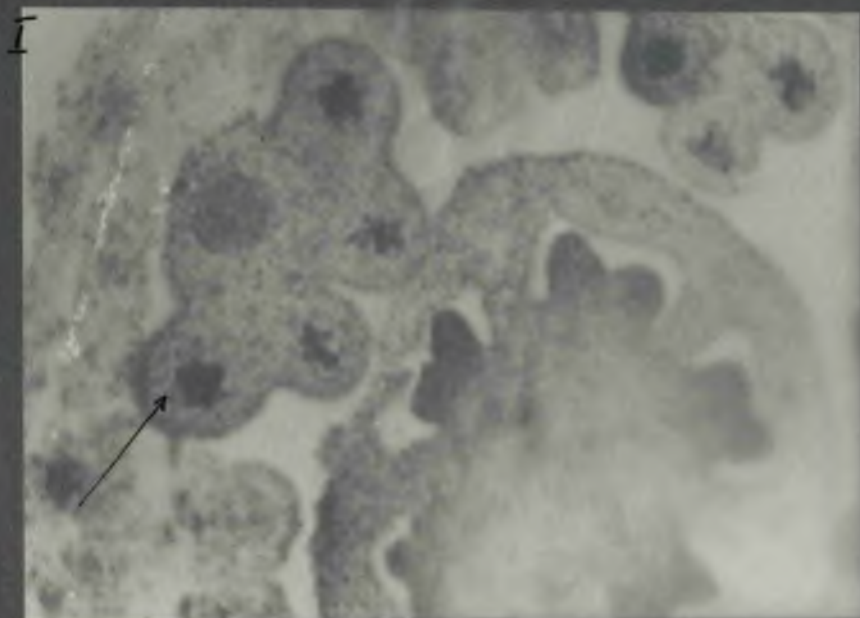
Fig. I.

A cross section through an infected bud from a female plant showing mycelium in the four anther chambers.

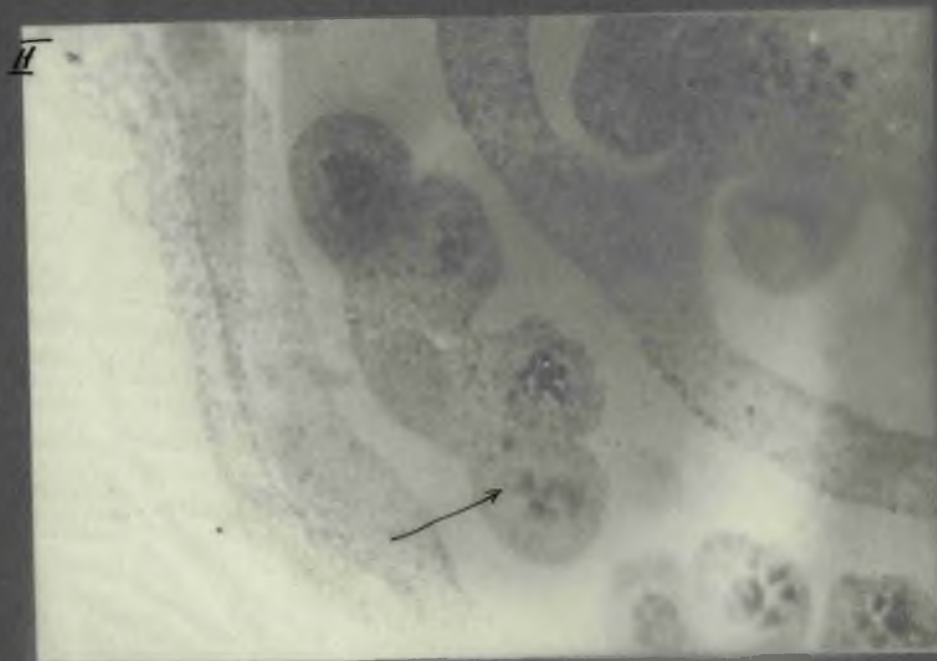
Fig. II.

A cross section through a younger infected bud from a female plant. The mycelium in the anther chambers is more peripheral.





40 μ.



25 μ

Fig. I.

A cross section through an infected bud from a female plant showing mycelium in the four anther chambers.

40x [ ]

Fig. II.

A cross section through a younger infected bud from a female plant. The mycelium in the anther chambers is more peripheral.

42x [ ]



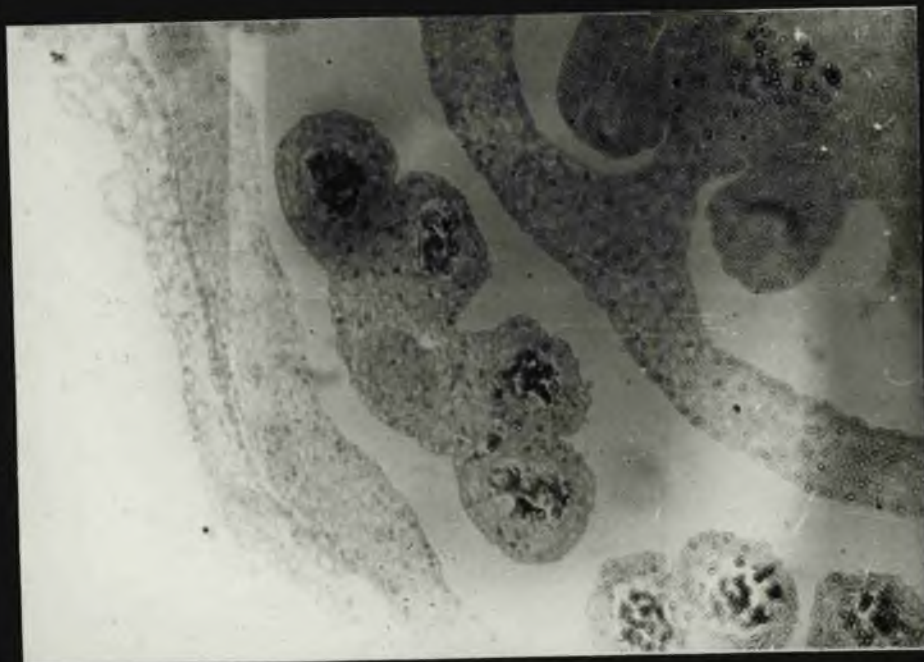
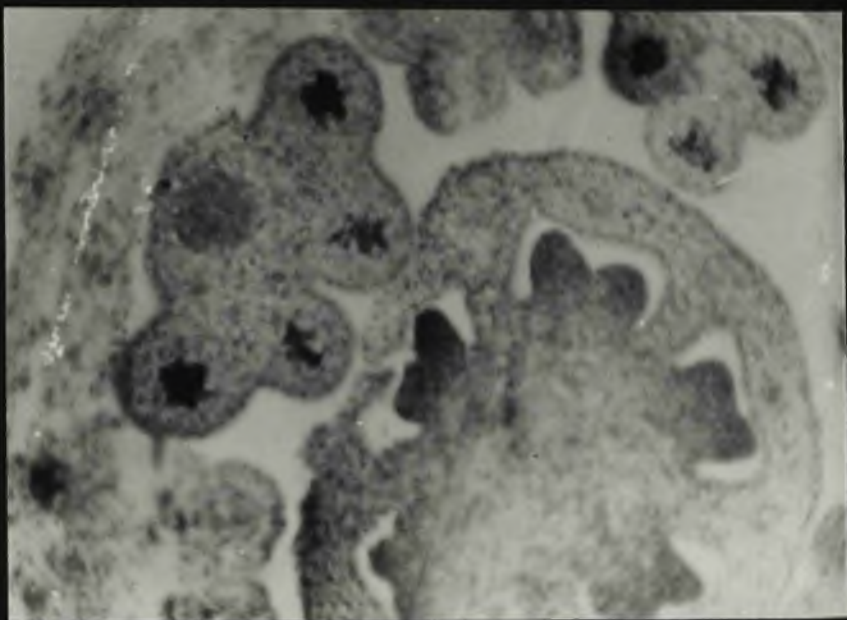


PLATE XV

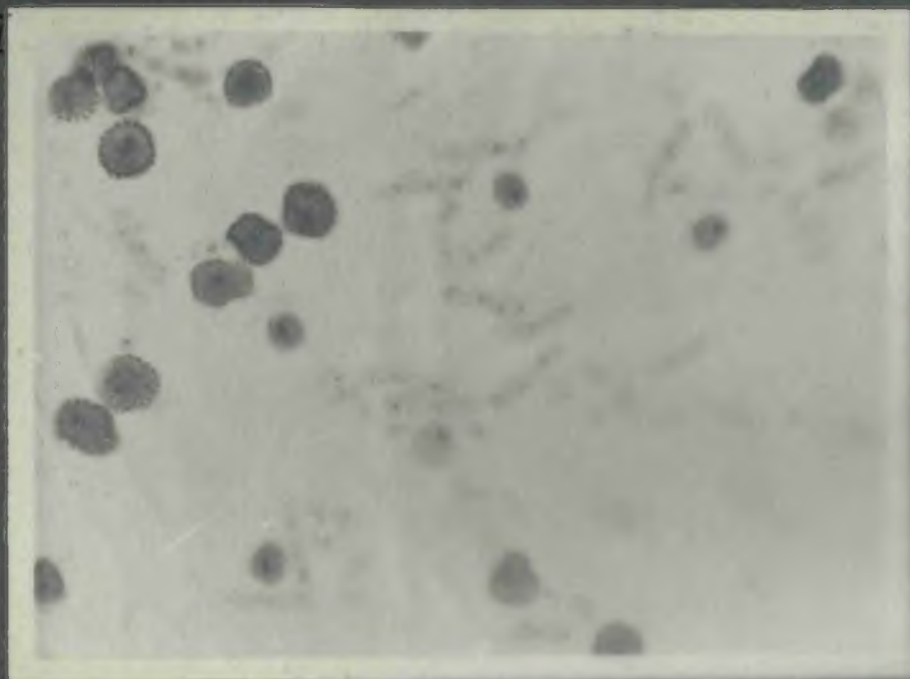
Fig. I.

Chlamydospores, promycelia, and sporidia  
illustrating their nuclear phenomena.  
( Newcomer's fixative and Azure A. )

Fig. II.

Chlamydospores, promycelia, and sporidia  
illustrating their nuclear phenomena.  
Terminal cells of promycelia joined (→),  
sporidia fusing without nuclear migration  
(↗).





┌ 10μ.

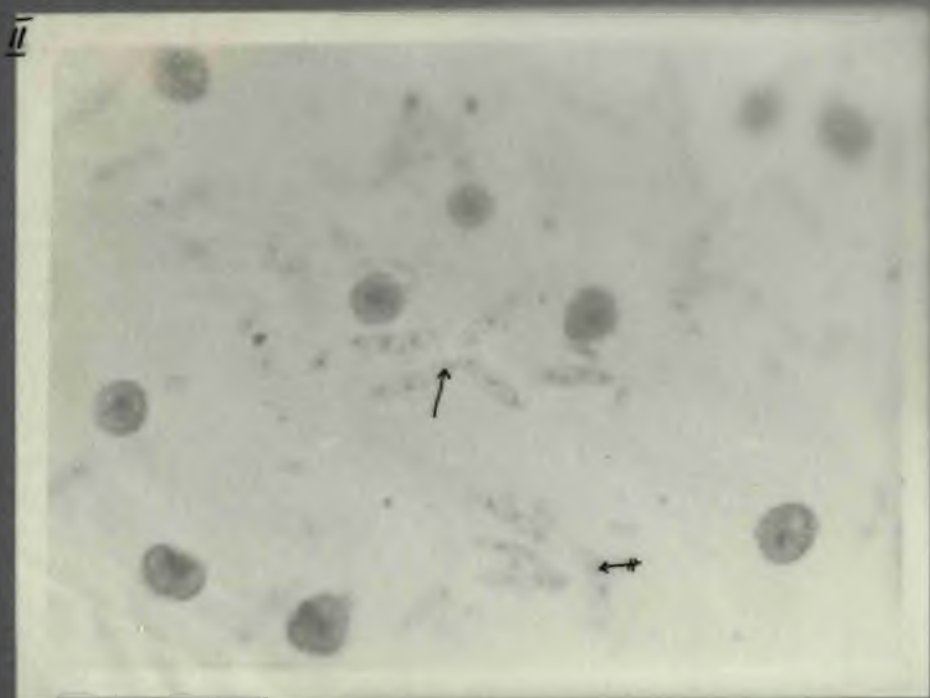


Fig. I.

Chlamydospores, promycelia, and sporidia  
illustrating their nuclear phenomena.  
( Newcomer's fixative and Azure A. )

40x [ ]

Fig. II.

Chlamydospores, promycelia, and sporidia  
illustrating their nuclear phenomena.  
Terminal cells of promycelia joined (↗),  
sporidia facing without nuclear migration  
(↖).

↑

↔



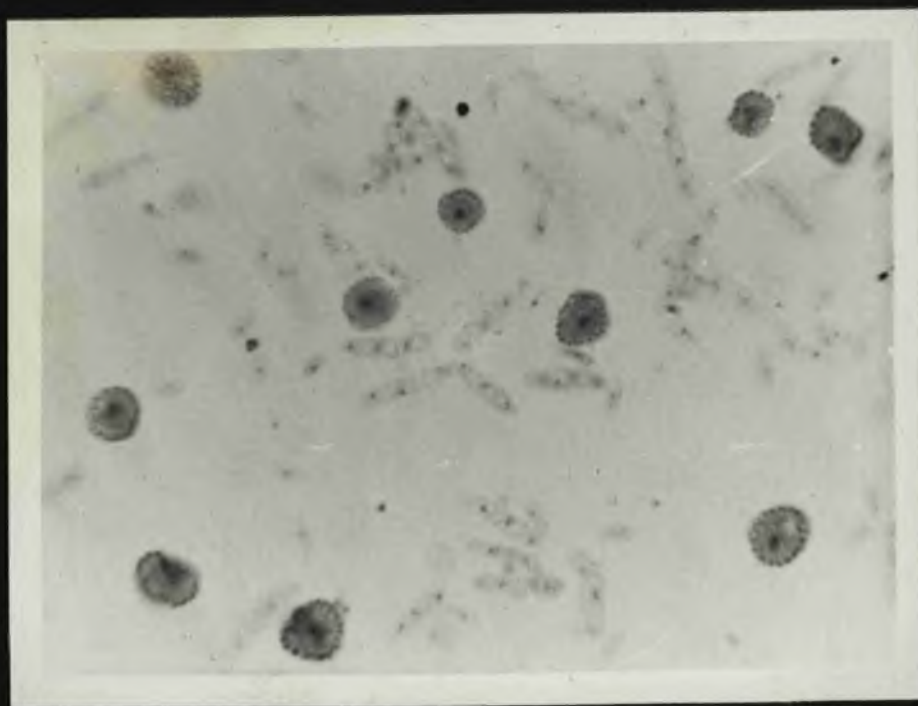
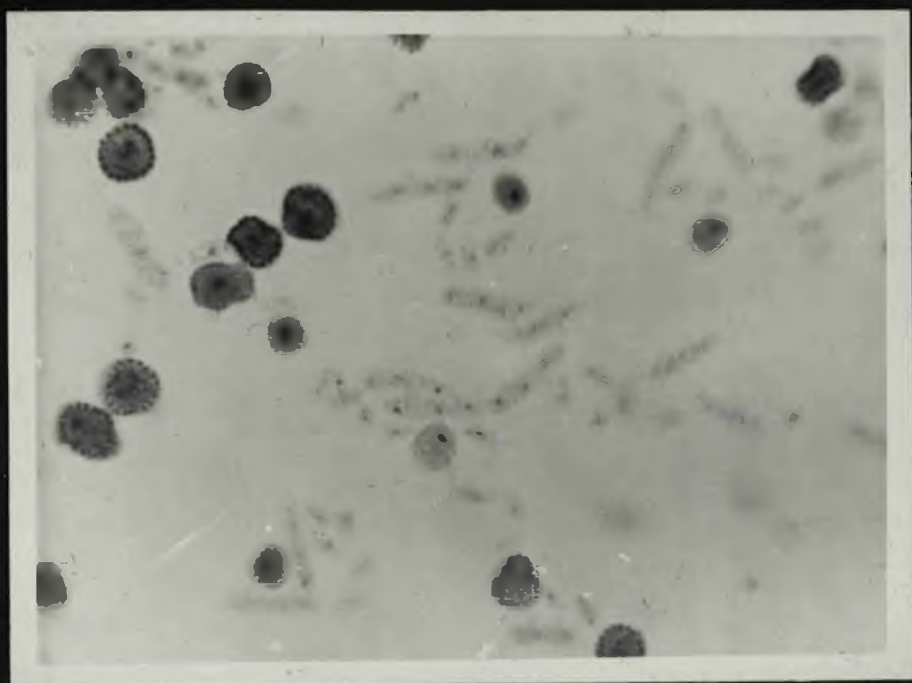


PLATE XVI

Fig. I.

- a) Nucleus dividing in the chlamydospore at the on-set of germination ( a germ tube is just emerging from the spore. ) Four chromosomes can be identified. ( ↗ )
- b) Free hand drawing showing the shape of the chromosomes.

( Newcomer's fixative & Azure A )

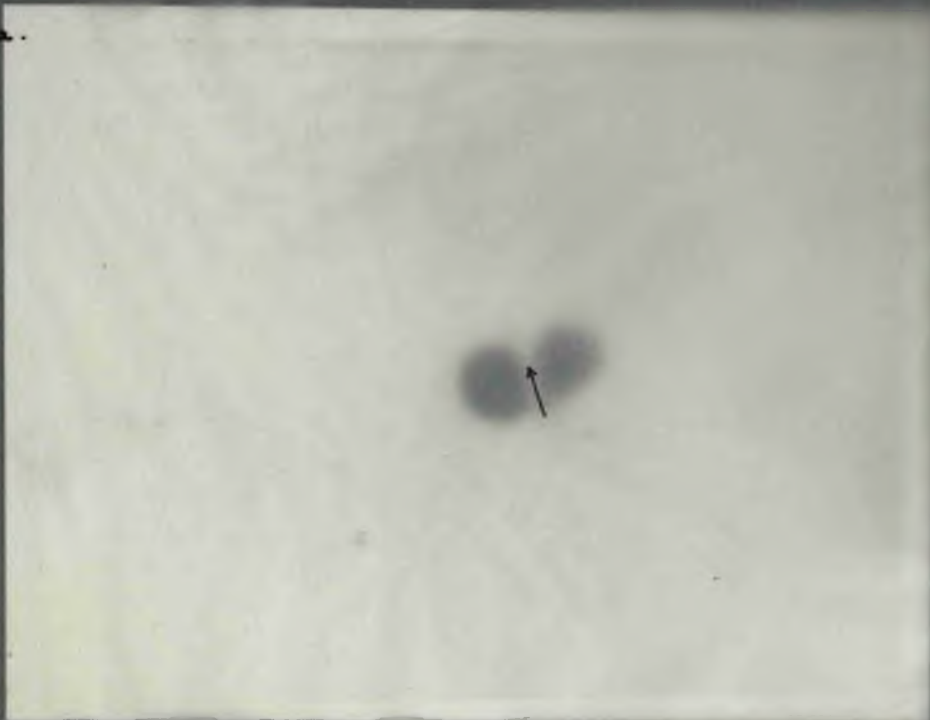
Fig. II.

- a) Germ tube forming two promycelial cells. The nucleus has divided mitotically into two nuclei each with two chromosomes. ( ↗ )
- b) Free hand drawing of the features in (a) above.

( Newcomer's fixative & Azure A )



Ia.

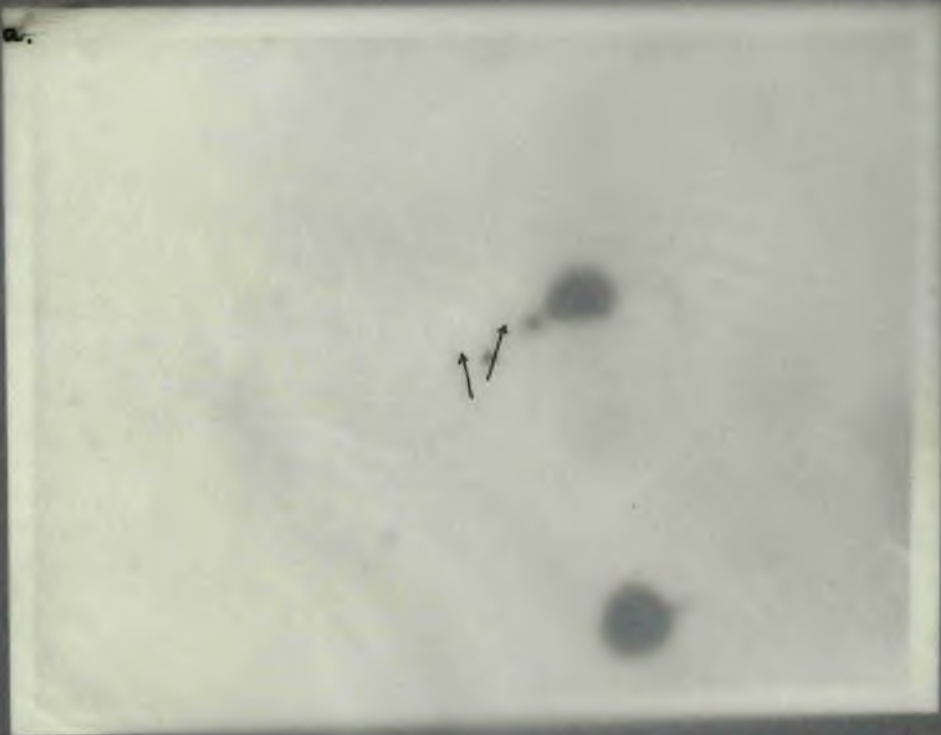


Ib.



10μ.

IIa.



IIb.



11

Fig. I.

- a) Nucleus dividing in the chlamydospore at the on-set of germination ( a germ tube is just emerging from the spore. ) Four chromosomes can be identified. ( ↗ )
- b) Free hand drawing showing the shape of the chromosomes.

( Newcomer's fixative & Azure A )

10/11

Fig. II.

- a) Germ tube forming two promycelial cells. The nucleus has divided mitotically into two nuclei each with two chromosomes. ( ↗ )
- b) Free hand drawing of the features in (a) above.

11

( Newcomer's fixative & Azure A )



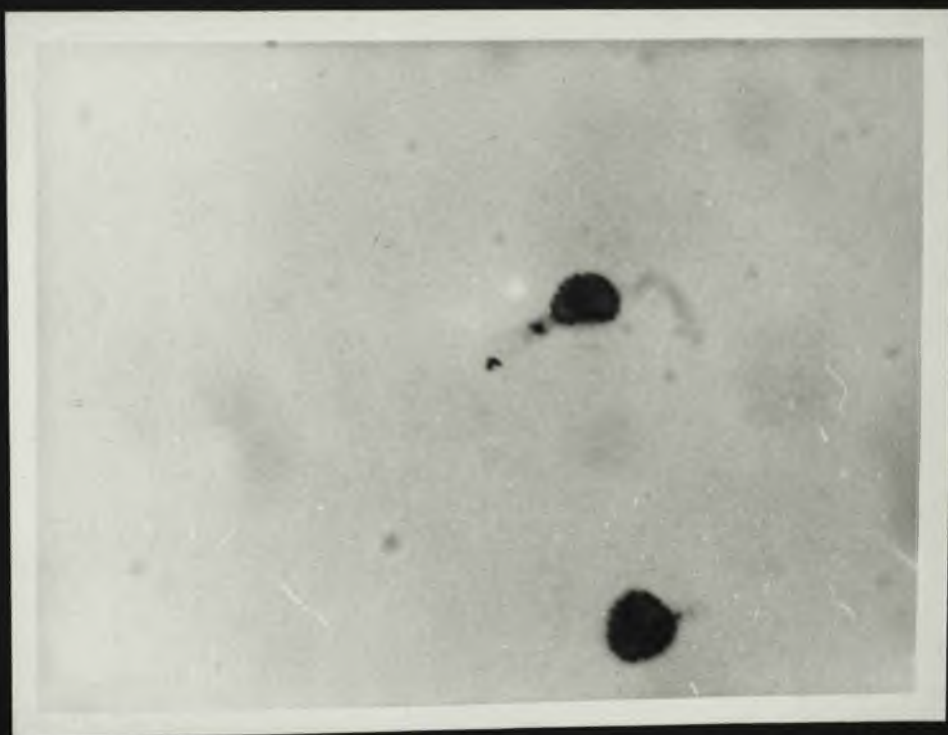


PLATE XVII

Fig. I.

Metaphase chromosomes of a female plant.

( Root-tip squashes with the Feulgen Method. )

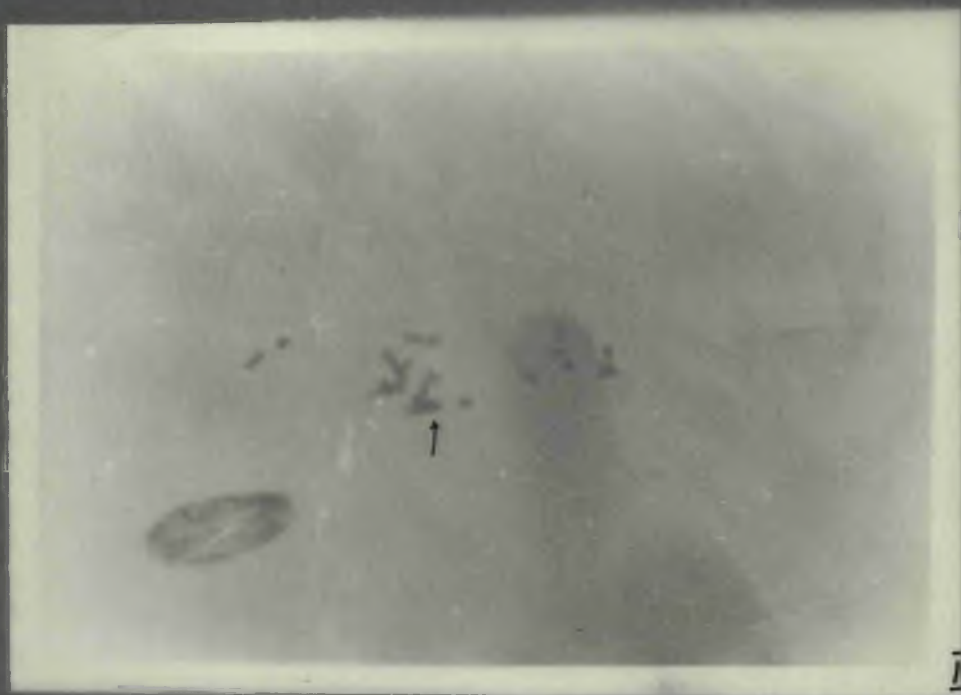
Fig. II.

Metaphase chromosomes of a male plant.

( Root-tip squashes with the Feulgens Method.)

- Y-sex chromosome(↗).





5/16.




Fig. I.

Metaphase chromosomes of a female plant.  
( Root-tip squashes with the Feulgen Method. )

.42 

Fig. II.

Metaphase chromosomes of a male plant. II  
( Root-tip squashes with the Feulgen Method. )  
- Y-sex chromosome (  ).

↓



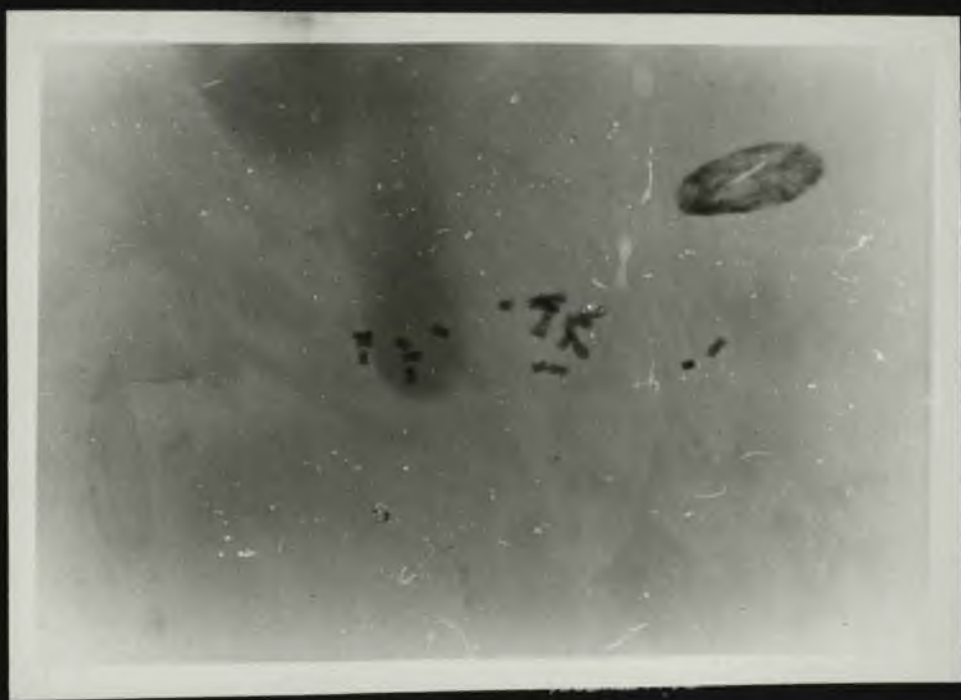


PLATE XVIII

Fig. I.

Metaphase chromosomes of a male plant.

( Root-tip squashes with propionic orcein stain.)

Fig. II.

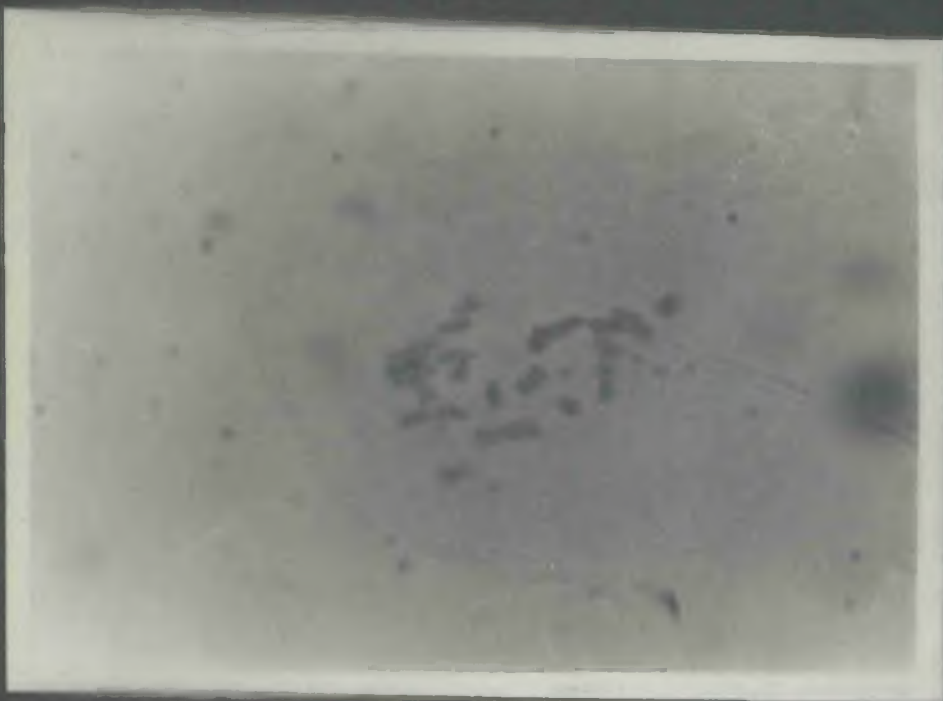
Metaphase chromosomes of one of the two plants showing female flowers as leaders in every branch— other flowers were male.

X & Y chromosomes (↗↘)

( Root-tip squashes with propionic orcein.)



I



5 $\mu$ .

II

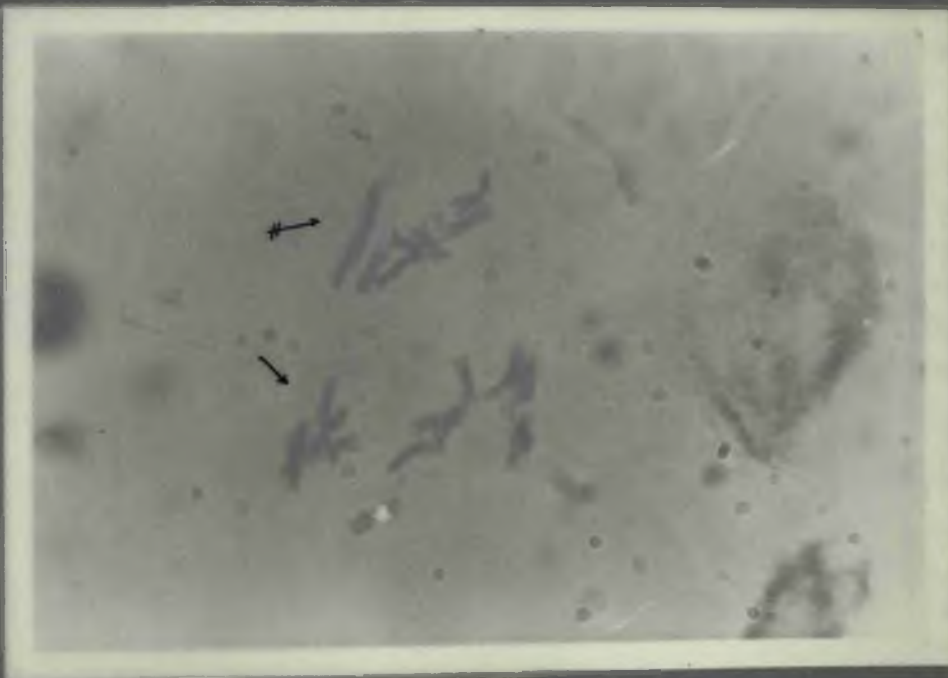


Fig. I.

Metaphase chromosomes of a male plant.

( Root-tip squashes with propionic orcein stain.)

.42 

Fig. II.

Metaphase chromosomes of one of the two plants  
showing female flowers as leaders in every  
branch— other flowers were male.

X & Y chromosomes (↗↘)

( Root-tip squashes with propionic orcein.)

↖

↙



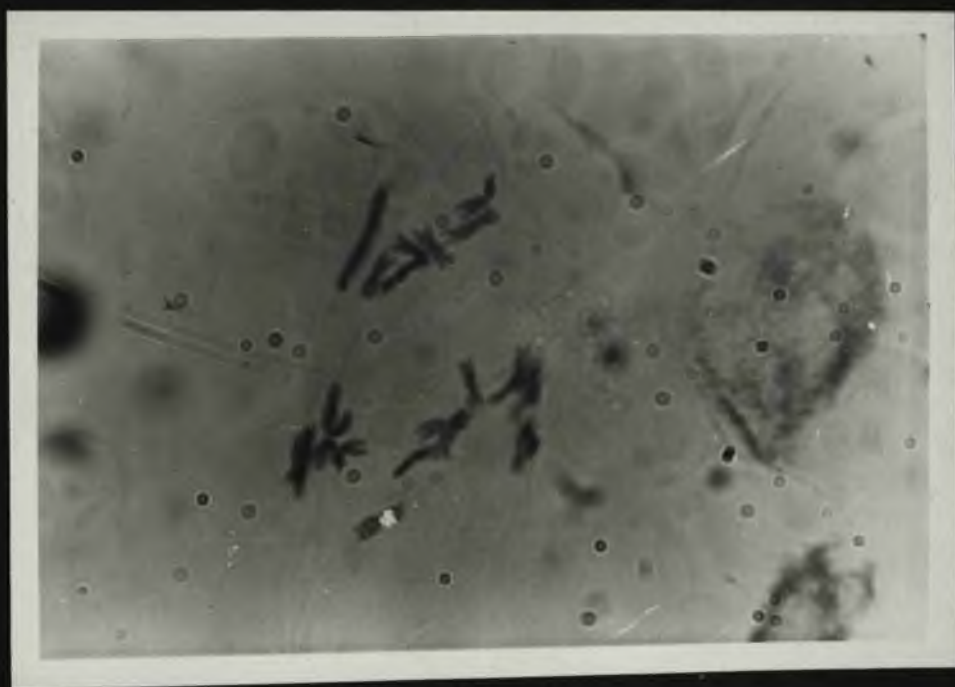
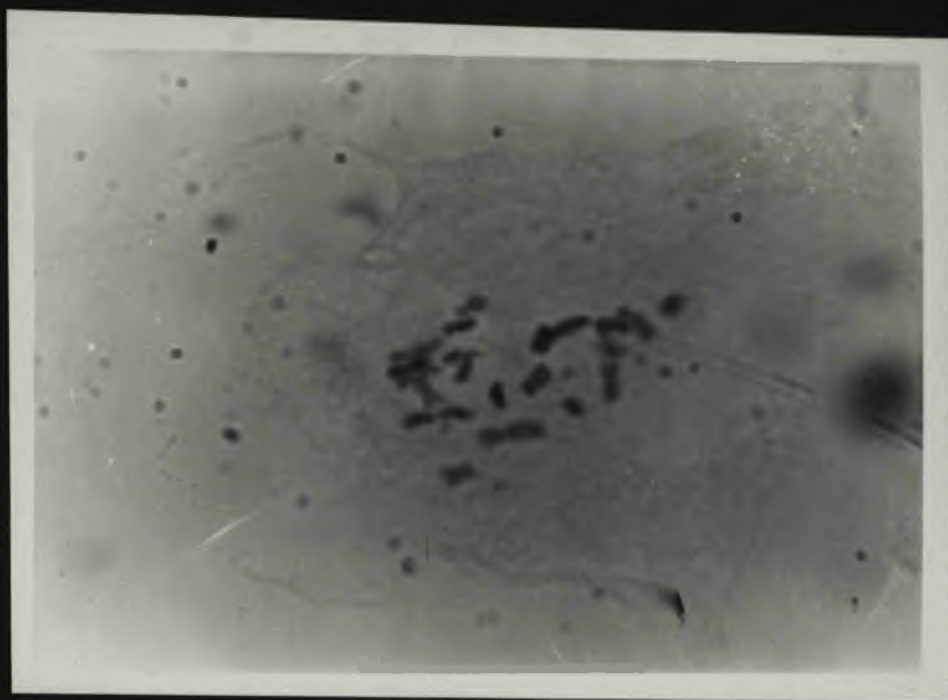


PLATE XIX

Fig. 1.

Diagram of the apparatus used in growing roots from plant cuttings where the roots were then employed in root-tip squashes in diseased plants.



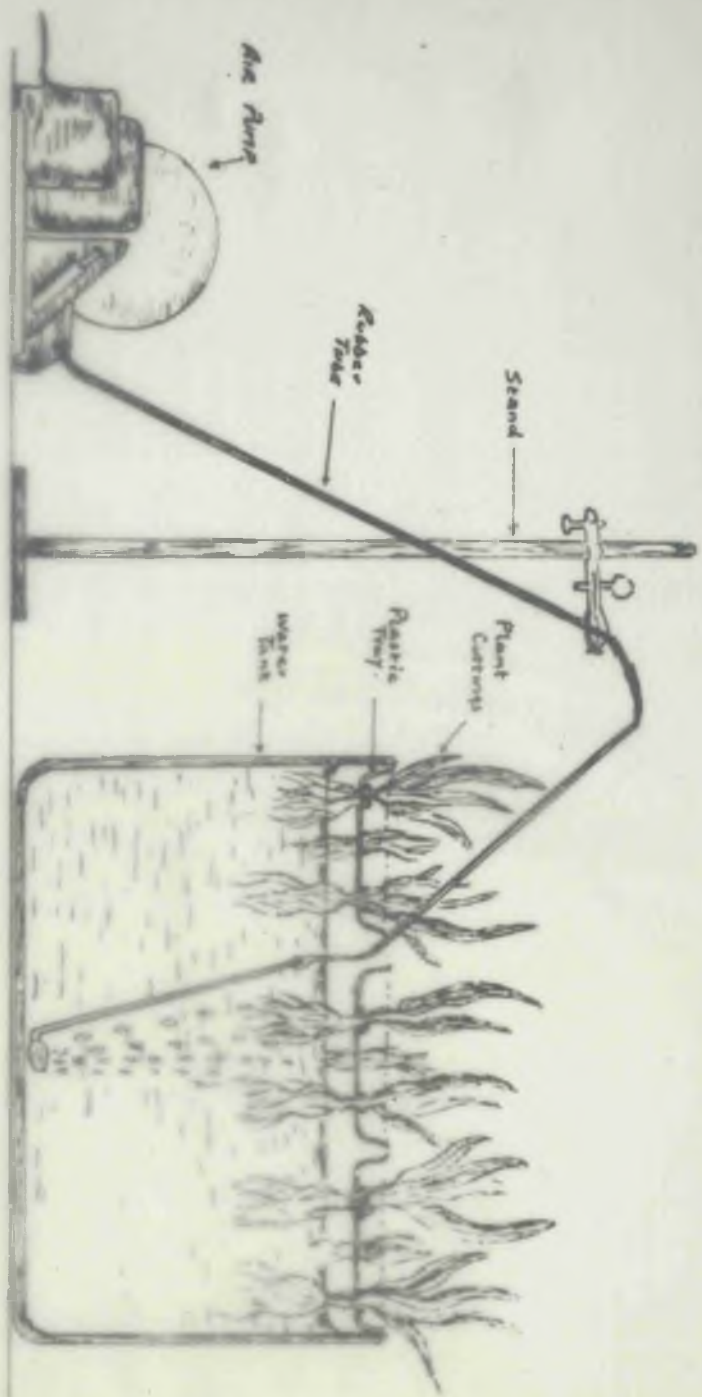


PLATE XIX

Fig. I.

Diagram of the apparatus used in growing roots from plant cuttings where the roots were then employed in root-tip squashes in diseased plants.



